Heterologous Expression and Site-Directed Mutagenesis of Soluble Methane Monooxygenase

John S. Lloyd B.Sc. (Hons) London

A thesis submitted for the degree of Doctor of Philosophy

Department of Biological Sciences

University of Warwick

Coventry

CV4 7AL

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DECLARATION

I declare that all of the work reported in this thesis, is the result of original research conducted by myself (under the supervision of Professor J. C. Murrell and Professor H. Dalton FRS). Additional help and information, when obtained, has been referenced.

No work contained within this thesis has been previously submitted for any other degree.

John S. Lloyd

ABBREVIATIONS

μ	Specific growth rate
Ω	Ohm (unit for electrical resistance)
A	Absorbance
AMO	Ammonia monooxygnease
Ap	Ampicillin
AMPS	Ammonium persulphate
bp	Base pairs
BPH	Biphenyl dioxygenase
BHR	Broad host range
Cm	Chloramphenicol
DCM	Dichloromethane
DEAE	Diethylamino Ethyl
DTT	D, L-dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EPR	Electron paragmagnetic resonance
ESI-MS	Electrospray ionisation-mass spectrometry
EXAFS	Extended X-ray absorption fine structure
FDH	Formate dehydrogenase
FADH	Formaldehyde dehydrogenase
FPLC	Fast protein liquid chromatography
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GST	Glutathione S-transferase
h	Hour
HPLC	High pressure liquid chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilo base pairs
kDa	Kilo Daltons
Km	Kanamycin
K_m	Michaelis constant
l	litre
Mc.	*Methylococcus*
Mcy.	*Methylocystis*
M	Molar
MDH	Methanol dehydrogenase
MPa	Mega pascals
mg	Milligram
min	Minute
ml	Millilitre
Mm.	*Methylomicrobium*
Ms.	*Methylosinus*
MMO	Methane monooxygenase
mol	Mole
mM	Millimolar

MOPS	3-[N-Morpholino] propanesulfonic acid
mRNA	Messenger ribonucleic acid
NA	Nutrient agar
NAD/NADH	Nicotinamide adenine dinucleotide (oxidised/reduced)
NCIMB	National Cultures of Industrial and Marine Bacteria
ND	Not determined
NH	No hybridisation
nm	Nanometre
NMS	Nitrate Minimal Salts
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pMMO	Particulate methane monooxygenase
pmol	Picomole
PMSF	Phenylmethylsulfonylfluoride
PQQ	Pyrroloquinoline quinone
RPM	Revolutions per minute
RuMP	Ribulose monophosphate
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sm	Streptomycin
sp.	Species
sMMO	Soluble methane monooxygenase
Tc	Tetracycline
Tg	Teragram
TCE	Trichloroethylene
TDO	Toluene dioxygenase
TEMED	*N, N, N', N'*-tetramethylethylenediamine
TOD	Tetrazotised-O-dianisidine
U.S.E	Unique Site Elimination
UV	Ultraviolet
WH	Weak hybridisation
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

SUMMARY

The purpose of this investigation was to study the heterologous expression of soluble methane monooxygenase (sMMO) genes from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. Using the T7-RNA polymerase expression system, the entire sMMO operon and subclones (constructed using the polymerase chain reaction) were over-expressed in *E. coli*. Results obtained using the *Mc. capsulatus* (Bath) sMMO operon confirmed previous reports (C. West, G. P. C. Salmond, H. Dalton and J. C. Murrell (1992). *J. Gen. Microbiol. 138*, 1301-1307) that functional expression of protein B and the reductase occurred but the hydroxylase was inactive. Similar results were obtained by expressing the sMMO operon of *Ms. trichosporium* OB3b in *E. coli*, using plasmids previously described (D. Jahng and T. K. Wood (1994). *Appl. Environ. Microbiol. 60*, 2473-2482). Protein B, the reductase and *orfY* were over-expressed and purified from *E. coli* using glutathione-S-transferase fusion proteins and affinity chromatography.

The expression of sMMO genes from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b was studied in *Pseudomonas putida*. A previous report (D. Jahng and T. K. Wood (1994). *Appl. Environ. Microbiol. 60*, 2473-2482) had suggested that functional expression of sMMO from *Ms. trichosporium* OB3b was achieved in *P. putida* F1. Attempts to repeat this work proved that protein B and the reductase were functionally expressed, but the hydroxylase was inactive. Similar results were obtained for the heterologous expression of the sMMO operon from *Mc. capsulatus* (Bath) in *P. putida*.

Methanotrophs were used for the heterologous expression of sMMO *via* two strategies. (1) The expression of sMMO from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b was studied in *Methylomonas album* BG8 and *Methylocystis parvus* OBBP. These are methanotrophs that do not express sMMO, but express particulate MMO (pMMO) only, to utilise methane as a sole carbon and energy source. Functional expression of the sMMO operon of *Ms. trichosporium* OB3b was achieved in *Mm. album* BG8, however, recombinant sMMO enzyme activity was poor and problems were encountered with the growth of the sMMO positive transconjugant methanotrophs. (2) sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b (H. Martin and J. C. Murrell (1995). *FEMS Microbiol. Letts. 127*, 243-248) were complemented with plasmid encoded genes and functional sMMO expression was obtained. Southern hybridisation analysis revealed that the plasmid DNA had integrated into the chromosome of the *Ms. trichosporium* OB3b sMMO-minus mutant *via* a single homologous recombination event between the *mmoX* genes.

Protein B from *Mc. capsulatus* (Bath) is inactivated by proteolysis to give rise to a truncated form designated protein B'. The Met12-Gly13 cleavage site was modified by site-directed mutagenesis to Met12-Gln13 which improved the stability of the protein when incubated at room temperature. Only after prolonged incubation was protein B' formed. Recombinant protein B from *Ms. trichosporium* OB3b also appears to be unstable, and readily degraded when incubated at room temperature. The cleavage of protein B to inactive protein B' may be a general regulatory mechanism that occurs within the cell to regulate sMMO activity.

CHAPTER 1

INTRODUCTION

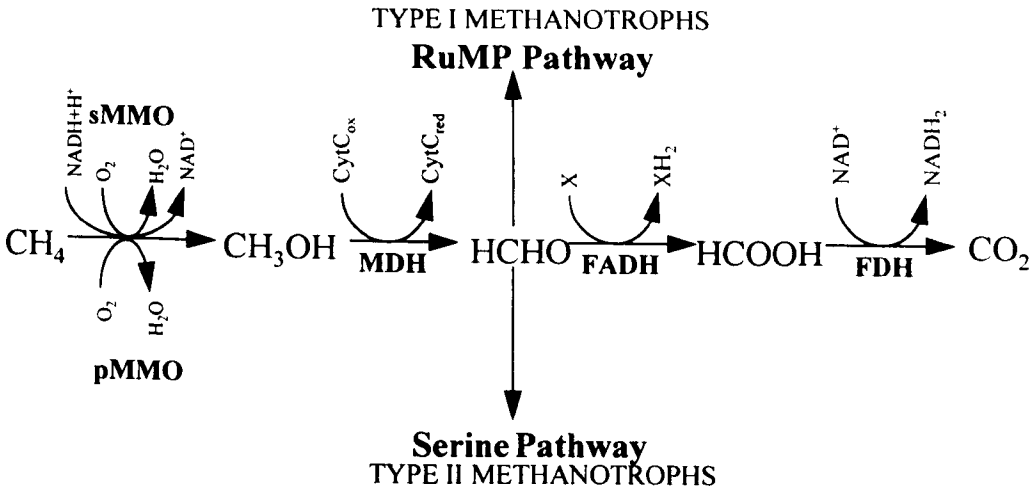
1.1 Methane utilising bacteria

Methane is the most abundant organic gas in the atmosphere (Crutzen, 1991) and the total sources of atmospheric methane have been estimated at 520 Tg per year. About 90 % of the methane is oxidised by photochemical reactions initiated by OH radicals, whilst a smaller amount (about 10 Tg $year^{-1}$) is lost by microbial oxidation in soils (Conrad, 1996). Atmospheric methane concentrations have increased during the past 300 years, most likely as a consequence of human activities. It has been predicted that increasing methane concentrations in the atmosphere will decrease OH radical concentrations and thus increase the lifetime of methane in the atmosphere (Lelieveld *et al.*, 1993). Since methane absorbs terrestrial radiation in the 4 to 100 nm region (infrared irradiation), re-emission of the absorbed radiant energy causes global warming. Reductions in methane emissions would be the most effective way of reducing the potential warming of the atmosphere over the next century (Hogan *et al.*, 1991). It is therefore important to elucidate the sources and sinks of atmospheric methane to determine which steps have practical use for reducing the global warming effects of this gas.

Methanotrophic bacteria are unique in their ability to utilise methane as a sole carbon and energy source (reviewed by Hanson and Hanson, 1996). They are a subset of a physiological group of bacteria known as methylotrophs (Anthony, 1982; Hanson, 1992). These aerobic bacteria are capable of utilising one-carbon compounds as sole carbon and energy sources and assimilate formaldehyde as a major source of cellular carbon. Methylotrophic bacteria utilise a range of one-carbon compounds including methane, methanol, methylated amines and halomethanes; however, the ability of organisms to oxidise methane to methanol *via* methane monooxygenase

(MMO) is the defining characteristic of the methanotrophs. The methane oxidation pathway utilised by the methanotrophs is shown in Figure 1.1.

Figure 1.1: Methane oxidation pathway. MDH, methanol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; pMMO, particulate MMO; sMMO, soluble MMO; RuMP, ribulose monophosphate. Adapted from Hanson and Hanson (1996).



The common features of methanotroph metabolism include the central role of formaldehyde as an intermediate in catabolism and anabolism, in addition to the unique pathways used for the synthesis of metabolic intermediates. Since an increase in atmospheric methane concentration is the prime cause of an increased rate of global warming, there is considerable interest in methanotrophs. Extensive studies have been concerned with determining the roles of methane-utilising bacteria in the regulation of methane emissions to the atmosphere and to illustrate the environmental

factors that affect the ecology of these organisms (reviewed by Hanson and Hanson, 1996). Commercial applications of the methanotrophs are discussed in Section 1.12.

There is extensive evidence to suggest that methane is also consumed in anaerobic environments including marine and freshwater sediments (King, 1992; Hanson and Hanson, 1996). To date, pure cultures of anaerobic methane oxidisers have not been isolated and so the biochemistry of this process remains to be elucidated.

1.2 Taxonomy of methanotrophs

In 1970, Whittenbury *et al.* isolated and characterised over 100 new methane-oxidising bacteria which have formed the basis for current classification schemes. The methanotrophs were classified into five groups based upon morphology, membrane structure, types of resting stages formed and some physiological characteristics (Whittenbury *et al.*, 1970). The genera, *Methylomonas* (*Mm.*), *Methylobacter* (*Mb.*), *Methylococcus* (*Mc.*), *Methylocystis* (*Mcy.*) and *Methylosinus* (*Ms.*) that were originally proposed are similar to those currently in use, except for the addition of one new genus, *Methylomicrobium* (*Mm.*) (Bowman *et al.*, 1993, 1995). The methanotrophs have been classified into three groups type I, type II and type X. Type I includes the genera *Mm.* and *Mb.* and type II includes the genera *Ms.* and *Mcy.* The new group type X, was created to include methanotrophs similar to *Mc. capsulatus* (Bath) which for formaldehyde assimilation, utilised the RuMP pathway found in type I methanotrophs (Table 1.1). Type X methanotrophs were distinguishable from type I methanotrophs because they possessed enzymes of the serine pathway, they grew at higher temperatures than type I and type II

Table 1.1: Characteristics of type I, type X and type II methanotrophs. Adapted from Green (1992).

Characteristic	Type I	Type X	Type II
Membrane arrangement			
Bundles/vesicular disks	+	+	-
Paired peripheral membranes	-	-	+
Motility	+	-	+
Resting cells	cyst	cyst	cyst or exospore
Carbon assimilation pathway	RuMP	RuMP	Serine
Autotrophic CO_2 fixation	-	+	-
Complete TCA cycle	-	-	+
Nitrogenase	-	+	+
Isocitrate dehydrogenase[1]			
NAD^+ and $NAD(P)^+$ specific	+	-	-
NAD^+ specific	-	+	-
$NAD(P)^+$ specific	-	-	+
Glucose-6-dehydrogenase	+	+	-[2]
Predominant fatty acid carbon chain length	16	16	18
Mol % G + C of DNA	50-54	62.5	61.7-63.1

[1] Not all Type I and Type II strains have been shown to possess all the biochemical characteristics.

[2] During methane-dependent growth.

methanotrophs and possessed DNA with a higher mol % G + C content than those of type I methanotrophs (Green, 1992; Hanson and Hanson, 1996).

Numerical taxonomic evaluation, phospholipid fatty acid analysis, DNA-DNA hybridisation and phylogenetic relationships have been used to clarify genus and species inter-relationships (Bowman *et al.*, 1993, 1995). The family *Methylococcaceae* was redefined to include the genera *Mc.*, *Mm.*, *Mb.* and *Mm.* In addition, the phylogenetic relationships of the methanotrophs have been investigated using 5S and 16S rRNA sequence analysis (Bulygina *et al.*, 1990; Bratina *et al.*, 1992; Bowman *et al.*, 1993, 1995). This work has shown that the methylotrophic bacteria which utilise the serine pathway for formaldehyde assimilation form a distinct group within the α subdivision of the Proteobacteria; the family *Methylococcaceae* form a distinct branch within the γ subdivision; the non-methane utilising methylotrophs that employ the RuMP pathway for formaldehyde assimilation are found within the α subdivision of the Proteobacteria.

1.3 Methane oxidation

The first reaction of methane oxidation is the oxidation of methane to methanol by MMO (Section 1.3.3). This is achieved using molecular oxygen and reduced NAD generated from formaldehyde and formate oxidation (Figure 1.1). MMO is the subject of this thesis and will be described in detail in Section 1.3.3.

1.3.1 Methanol dehydrogenase

In Gram negative methylotrophs, methanol is oxidised to formaldehyde by the periplasmic pyrroloquinoline quinone (PQQ)-dependent MDH (Davidson *et al.*, 1985;

Anthony, 1992; Anthony and Dales, 1996). MDH is an $\alpha_2\beta_2$ tetramer of large (60 to 70 kDa) and small (8.5 kDa) subunits. Electrons are transferred from MDH to cytochrome c_L, which is then re-oxidised by cytochrome c_H. The understanding of MDH has rapidly increased following the publication of the X-ray crystal structure of the enzymes from *Methylotrophus* W3A1 at 2.6 Å (Xia *et al.*, 1992) and *Methylobacterium extorquens* at 1.94 Å (Anthony and Dales, 1996). MDH from *Mb. extorquens* comprises a hydrophobic active site chamber containing a PQQ prosthetic group and an unusual disulphide bridge formed between adjacent cysteine residues. The rarity of the disulphide bridge between adjacent cysteine residues suggests that this disulphide bridge may play an important biological function in MDH. Recent studies suggest that it may serve to hold PQQ in place within the active site (Anthony and Dales, 1996). The genes encoding the large and small subunits of MDH and cytochrome c_L have been cloned from a variety of organisms including *Mb. extorquens* and *Paracoccus denitrificans* (Lidstrom, 1992; Harms, 1993) and methanotrophs including *Mc. capsulatus* (Bath) and *Mm. album* BG8 (Stephens *et al.*, 1988).

1.3.2 Formaldehyde and formate dehydrogenase

Most of the reducing power for the metabolism of methane is derived from the oxidation of formaldehyde *via* formate to carbon dioxide. Formaldehyde dehydrogenases (FDHs) from *Ms. trichosporium* OB3b (Patel *et al.*, 1980) and *Mm. album* BG8 (Leak and Dalton, 1983) have been shown to oxidise other aldehydes including straight chain aliphatic and aromatic aldehydes. Formate dehydrogenase

has been purified from *Ms. trichosporium* OB3b and consists of two subunits of about 103 and 54 kDa, in an $\alpha_2\beta_2$ configuration (Yoch *et al.*, 1990).

1.3.3 Methane monooxygenase (MMO)

Two forms of MMO are present in methanotrophs, particulate MMO (pMMO) and soluble MMO (sMMO). pMMO is membrane associated and so enzyme activity is identified in the insoluble fraction of cell free extracts. In contrast, sMMO is identified in the soluble fraction and is not membrane associated. The biochemistry and molecular biology of the MMOs are described in Sections 1.4 to 1.5. The copper-to-biomass ratio achieved under different growth conditions is responsible for determining the intracellular location of MMO activity. This will be discussed further in Section 1.9.

1.4 pMMO

1.4.1 Biochemistry of pMMO

Methane oxidation in membrane fractions has been reported in several methanotrophs including *Mm. methanica* (Ferenci, 1974; Colby *et al.*, 1975) and *Ms. trichosporium* OB3b (Tonge *et al.*, 1975). The major problem with studying pMMO has been the lack of a pure enzyme system since enzymatic activity is readily lost during the solubilisation and purification of the enzyme (Dalton, 1992). More recently, pMMO from *Mc. capsulatus* (Bath) has been extensively studied (Smith and Dalton, 1989; Shiemke *et al.*, 1995; Tukhvatullin *et al.*, 1996; Cook and Shiemke, 1996) culminating in an extensive characterisation of highly active pMMO-enriched

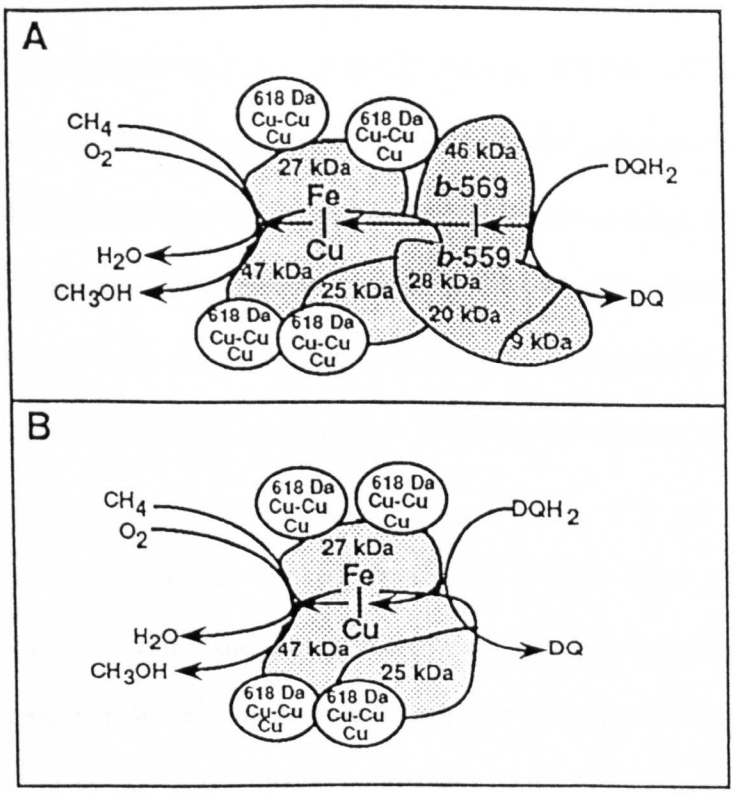
membranes (Nguyen *et al.*, 1996a,b) and purified pMMO (Zahn and DiSpirito 1996) from this organism.

pMMO enriched membranes contained major polypeptides of 46, 23 and 20 kDa and had a specific activity of 5-60 nmol min^{-1} mg $protein^{-1}$ (Nguyen *et al.* 1996b). Characterisation of the pMMO-associated copper ions showed that they exist in both Cu(I) and Cu(II) oxidation states in the membranes and were reactive towards dioxygen. Highly active preparations contained Cu(I) suggesting that the functional form of the enzyme must be fully- or partially-reduced. Based upon their observations, Nguyen *et al.* (1996a) proposed the following mechanism for pMMO. The fully-reduced copper cluster could bind dioxygen, which would be followed by a two electron reduction to form a copper-peroxy intermediate. Dioxygen bond cleavage would then generate a copper-oxygen intermediate capable of alkane hydroxylation.

Using a combination of ion exchange and hydrophobic interaction chromatography using dodecyl β-*D*-maltoside as the detergent, Zahn and DiSpirito (1996) obtained pure active preparations of pMMO. Three major polypeptides of 47, 27 and 25 kDa were identified with a specific activity of 11 nmol min^{-1} mg $protein^{-1}$. The proteins of 47 and 27 kDa were identified as the polypeptides induced when cells expressing sMMO were switched to culture media containing copper, required for the expression of pMMO. Acetylene is a suicide substrate for sMMO and pMMO (Prior and Dalton, 1985) and the 27 kDa protein of pMMO was identified as the acetylene binding protein. The same 27 kDa pMMO protein was also found to bind acetylene by Zahn and DiSpirito (1996). The active enzyme complex contained 2.5 iron atoms and 14.5 copper atoms per 99,000 Da. Electron paragmagnetic resonance (EPR) was

used to show that the non-heme iron of the purified pMMO was either a single Fe(II) centre, an Fe(II)-Fe(II) centre or a copper-Fe(II) centre. Pure pMMO also contained 3-7 mol % cytochrome *b*-559/569 and it was proposed that this could function as the initial electron donor to the pMMO. Based upon these results, Zahn and DiSpirito (1996) have proposed a model for the mechanism of pMMO (Figure 1.2). Although

Figure 1.2: Proposed mechanism of methane oxidation by pMMO using (a) cytochrome *b*-559/569 and (b) duroquinol as reductant. Reproduced from Zahn and DiSpirito (1996).



both iron and copper were proposed to be within the active site of pMMO, it was suggested that the majority of copper functioned to stabilise the enzyme.

pMMO shares many similarities with ammonia monooxygenase (AMO) found in the ammonia-oxidising nitrifying bacteria both at the protein and genetic (Section 1.4.2) level. The two enzymes are both membrane associated, have broadly similar substrate specificities and can both oxidise ammonia and methane (although the apparent K_m values are different). pMMO and AMO are inhibited by acetylene and ^{14}C-acetylene binds to the 27 kDa membrane-bound protein of pMMO in *Mc. capsulatus* (Bath) (Prior and Dalton, 1985) and the corresponding polypeptide of AMO from *Nitrosomonas europaea* (McTavish *et al.*, 1993). Copper ions stabilise pMMO (Zahn and DiSpirito, 1996) and AMO (Ensign *et al.*, 1993). Evidence suggests that AMO from *Nm. europaea* contains an iron centre (Zahn *et al.*, 1996) consistent with the observations made for pMMO (Zahn and DiSpirito, 1996).

In whole cells of *Ms. trichosporium* OB3b, pMMO showed a much narrower substrate specificity than sMMO since it was unable to oxidise aromatic or alicyclic compounds such as benzene, ethylbenzene, styrene and cyclohexane (Burrows *et al.* 1984). This narrow substrate specificity was subsequently confirmed by Nguyen *et al.* (1996b) using pMMO-enriched membranes. Only C1-C5 alkanes and alkenes were oxidised.

Results obtained to date on the purification of pMMO have presented a confusing picture of this enzyme and clearly there is still much more to learn. The discrepancies between the findings of Zahn and DiSpirito (1996) and Nguyen *et al.* (1996a,b) with regard to the metal content of pMMO await further investigations. This should be made more straight forward thanks to the recent advances in the isolation and purification of the enzyme.

1.4.2 Molecular biology of pMMO

Nothing was known about the molecular biology of pMMO until the cloning and sequencing of the genes encoding the putative 27 and 43 kDa polypeptides of AMO from *Nm. europaea*, designated *amoA* and *amoB* respectively (McTavish *et al.*, 1993). Oligonucleotide primers were designed in order to use the polymerase chain reaction (PCR) to amplify and clone a 693 bp internal DNA fragment of the *amoA* gene from *Nm. europaea*. Subsequently, an oligonucleotide probe was designed following the N-terminal sequencing of the putative 46 kDa polypeptide of pMMO from *Mc. capsulatus* (Bath). Hybridisation of these probes to chromosomal DNA digests of *Mc. capsulatus* (Bath) was used to identify a 2.1 kb region of DNA that contained *pmoA* and *pmoB* (Semrau *et al.*, 1995). The predicted amino acid sequences of these genes revealed approximately 43 % and 47 % identity with the corresponding *amoA* and *amoB* gene products respectively. The N-terminal amino acid sequences of the 47 and 27 kDa pMMO proteins were consistent with the DNA sequence of *pmoA* and *pmoB* respectively (Zahn and DiSpirito, 1996). The presence of two hybridising bands in methanotrophs suggested that duplicate copies of *pmoA* and *pmoB* might exist, an observation consistent with duplicate gene copies of *amoA* and *amoB* from *Nm. europaea* (McTavish *et al.*, 1993).

By sequencing upstream of *pmoA*, Costello *et al.* (1995) identified a third open reading frame, designated *pmoC* which encodes the 25 kDa protein of pMMO (confirmed by comparing the N-terminal amino acid sequence with the deduced amino acid sequence) and was present in duplicate copies. In addition, Costello *et al.* (1995) used PCR-mediated cloning (based upon the pMMO DNA sequence from *Mc. capsulatus* (Bath)) to clone and sequence *pmoA* and *pmoB* from *Mm. album* BG8.

Genes encoding *pmoA* and *pmoB* have also been cloned and DNA sequenced from *Ms. trichosporium* OB3b (Finch, 1997). PmoA and PmoB from *Ms. trichosporium* OB3b have a 57 % amino acid identity (82 % amino acid similarity) and 45 % identity (62 % amino acid similarity) to the equivalent proteins from *Mc. capsulatus* (Bath).

Consistent with the observation of duplicate genes, Lidstrom and Semrau (1995) have proposed that the pMMO can exist in two kinetically and spectroscopically distinct forms, depending on the copper concentration in the growth medium. At a high copper concentration, pMMO with a low K_m is expressed whilst at low copper concentrations, pMMO with a high K_m is expressed. There is the possibility that the two forms of pMMO are expressed from different operons.

In *Mm. album* BG8, Semrau *et al.* (1995) showed that separate weak and strong hybridising bands were identified when *Mc. capsulatus* (Bath) *pmoB* was used as a probe. This suggested that one of the two copies of *pmoB* was more similar to *Mc. capsulatus* (Bath) than the other. The same DNA fragment appeared to be more similar to *amoA* from *Nm. europaea* than the *pmoA* of *Mc. capsulatus* (Bath). This provided the first evidence that the two sets of pMMO genes had diverged within the methanotrophs and that pMMO and AMO were closely related at the genetic level. By designing degenerate oligonucleotide primers based on regions of shared amino acid sequence between the 27 kDa polypeptide (containing the active site) of pMMO and AMO, genes encoding these polypeptides (*pmoA* and *amoA*) were amplified by PCR from methanotrophic and nitrifying bacteria (Holmes *et al.*, 1995; Murrell and Holmes, 1996). The overall amino acid sequence conservation between AmoA and PmoA was 40 % identity and 65 % similarity. Three groups representing the α, β and

γ subdivisions of the Proteobacteria were seen, rather than two groups representing AmoA and PmoA. This provided additional evidence that pMMO and AMO may have a common evolutionary origin despite their different roles in methanotrophs and ammonia-oxidising bacteria.

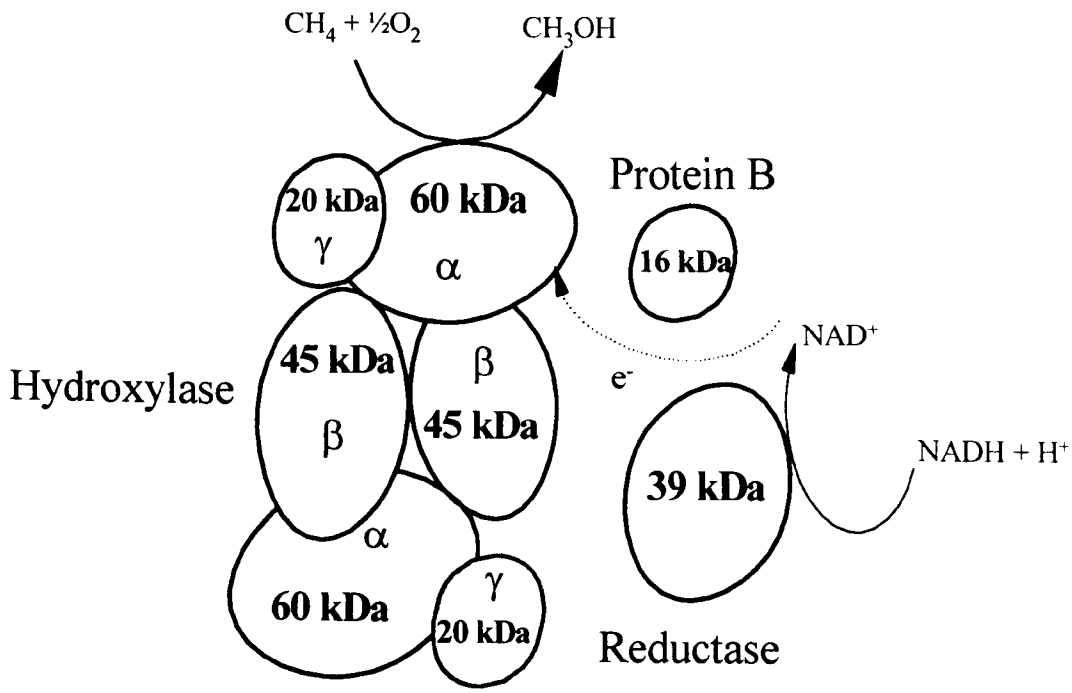
1.5 sMMO

1.5.1 Biochemistry of sMMO

sMMO has been purified from various methanotrophs including *Methylobacterium* sp. strain CRL-26 (Patel and Savas, 1987), *Ms. sporium* 5 (Pilkington and Dalton, 1991) and *Methylocystis* sp. strain M (Nakajima *et al.*, 1992), whilst it has been most extensively characterised from *Mc. capsulatus* (Bath) (Pilkington and Dalton, 1990) and *Ms. trichosporium* OB3b (Fox *et al.* 1989). It is now clear that sMMO comprises three subunits: the hydroxylase, protein B and the reductase (Figure 1.3). The only exception to this is sMMO from *Methylobacterium* sp. strain CRL-26 which does not appear to contain protein B. The hydroxylase contains a di-iron centre and comprises six subunits, forming an $\alpha_2\beta_2\gamma_2$ complex of about 250 kDa. Protein B is a single subunit protein of about 16 kDa with no metals or cofactors. The reductase is also a single subunit protein that has a molecular mass of about 40 kDa and contains FAD and a Fe_2S_2 cluster.

Each of the components of sMMO will be discussed in further detail, followed by a discussion of the proposed mechanism of methane oxidation by this enzyme, in light of recent work including the X-ray crystal structure of the hydroxylase component. Several comprehensive reviews on the biochemistry of sMMO are available, for example Dalton (1991, 1992), Dalton *et al.* (1993) and Lipscomb

Figure 1.3: Schematic diagram of sMMO.



(1994).

1.5.1.1 Hydroxylase

In *Mc. capsulatus* (Bath), the hydroxylase comprises three subunits, designated α, β and γ, of 54, 42 and 17 kDa, respectively. These are arranged in an $\alpha_2\beta_2\gamma_2$ configuration forming a complex of about 210 kDa (Woodland and Dalton, 1989). The protein was shown to contain 2.3 moles of non-heme iron per mole of protein. The hydroxylase component of *Ms. trichosporium* OB3b comprises three subunits α, β and γ of 54, 43 and 23 kDa respectively, arranged in an $\alpha_2\beta_2\gamma_2$ arrangement forming a complex of about 245 kDa (Fox *et al.*, 1989). The iron content of the enzyme was calculated as 4.3 moles of non-heme iron per mole of protein.

Woodland and Dalton (1989) used EPR to show that the hydroxylase contained Fe(III) and upon reduction with dithiothreitol (DTT), Fe(II) was identified. This was the first report to suggest that sMMO could contain a novel iron-containing prosthetic group. This was an unprecedented finding in an oxygenase, since similar structures had only been identified in hemerythrin, purple acid phosphatase and ribonucleotide reductase (Wilkins, 1992). Evidence that a μ-oxo binuclear-type bridge was present in the hydroxylase was confirmed using extended X-ray absorption fine structure (EXAFS) studies (Ericson *et al.*, 1988). This was also confirmed for the hydroxylase from *Methylobacterium* sp. strain CRL-26 (Prince *et al.*, 1988) and *Ms. trichosporium* OB3b (Fox *et al.*, 1988) using additional techniques including Mössbauer and EPR spectroscopy. X-ray crystal structure data have revealed that the di-iron centre forms a 'diamond core' with two bridging oxygen ligands (Rosenzweig *et al.*, 1993, 1995, 1996; Elango *et al.*, 1997).

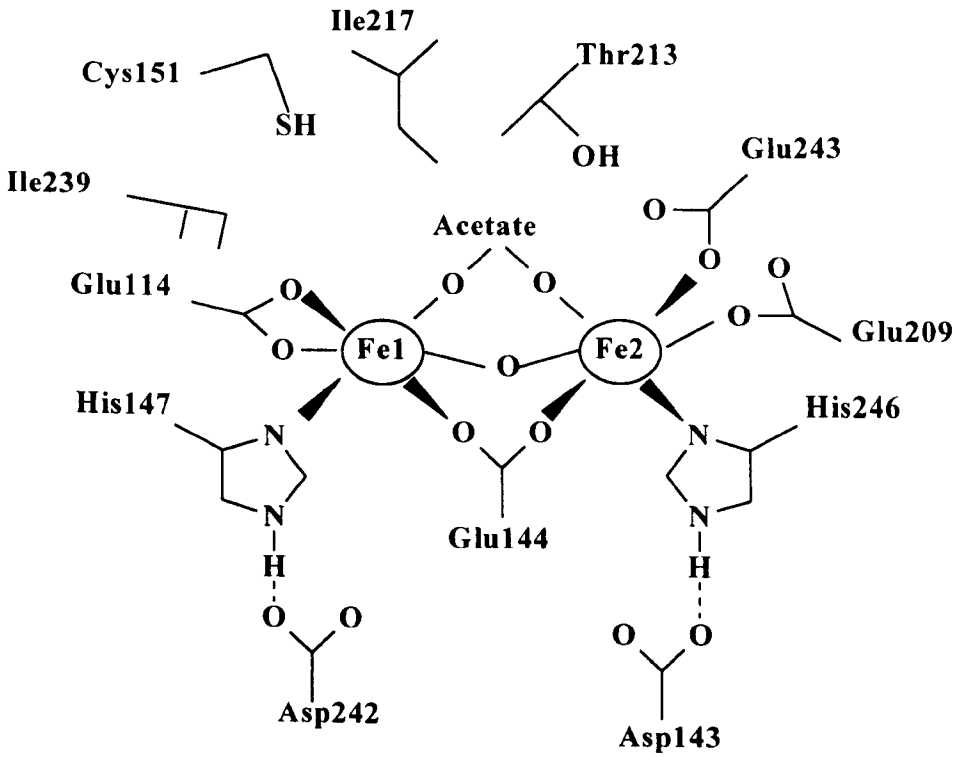
Green and Dalton (1988) showed that iron was required for catalysis by removing the iron centre of the hydroxylase by freeze/thaw cycles or by dialysis against 8-hydroxyquinoline. Incubation of the inactive, iron-depleted protein with iron-EDTA and DTT resulted in the re-assembly of the iron centre and a 3-fold increase in enzyme activity with respect to purified hydroxylase. Smith and Dalton (1992) used circular dichroism spectroscopy to show that the structures of the apoprotein and holoenzyme were the same. To determine the redox properties, any spectral changes and possible enzyme activity of the hydroxylase substituted with different metal ions, Mn(II) was incorporated into the apohydroxylase of *Mc. capsulatus* (Bath) (Atta *et al.*, 1993). Although this gave rise to inactive hydroxylase, it was concluded that because the apohydroxylase incorporated two Mn(II) ions and

lost 2 Fe(III) ions mol protein^{-1}, the hydroxylase of *Mc. capsulatus* (Bath) probably contained a single di-iron centre.

In vitro translation of total RNA isolated from *Mc. capsulatus* (Bath) produced the subunits of the hydroxylase equal to the native molecular mass, suggesting that no post-translational processing occurred (Green and Dalton, 1988).

The cloning and DNA sequencing of the genes encoding sMMO from *Mc. capsulatus* (Bath) (Stainthorpe *et al.*, 1989, 1990) and *Ms. trichosporium* OB3b (Cardy *et al.*, 1991a,b) revealed that the active site structures of the hydroxylase of the sMMOs were similar to the R2 subunit of *Escherichia coli* ribonucleotide reductase based upon amino acid sequence homologies. Within the binuclear iron-binding site of the R2 subunit of ribonucleotide reductase, a sequence motif of Glu-X-X-His was shown to coordinate the iron ions (Nordlund *et al.*, 1990a). The same sequence motif was identified in sMMO, suggesting that the principal ligands of the binuclear iron centres were similar. This information was used to propose the first model of the location of the principal ligands at the active site of sMMO (Murrell, 1992; Dalton, 1992; Nordlund *et al.*, 1992; Dalton *et al.*, 1993) and was consistent with findings that two histidine ligands were at the di-iron site of the enzyme (DeWitt *et al.*, 1991; Smith and Dalton, 1992) (Figure 1.4). X-ray crystal structures of the hydroxylase components of the sMMOs from *Mc. capsulatus* (Bath) (Rosenzweig *et al.*, 1993, 1995, 1996) and *Ms. trichosporium* OB3b (Elango *et al.*, 1997) have been solved to a resolution of 1.7 and 2.0 Å, respectively. The secondary structure of the hydroxylase was primarily helical. The coordination of the di-iron centre of the *Mc. capsulatus* (Bath) hydroxylase to the protein ligands was in agreement with the model proposed by Nordlund *et al.* (1992) and other workers (Figure 1.4). Since methane is such a

Figure 1.4: Proposed model for the active site structure of the sMMO hydroxylase from *Mc. capsulatus* (Bath). Adapted from Nordlund *et al.* (1993).



small non-polar molecule, the active site pocket of the hydroxylase component must be adapted to bind this substrate. A hydrophobic cavity was identified within the di-iron centre (Rosenzweig *et al.*, 1993; George *et al.*, 1996) which probably fulfils this role. In addition, there is no direct path to the active site from the surface of the protein, however, significant hydrophobic pockets occur in the α subunit which may provide a route for substrate to access the di-iron centre.

The structure of the hydroxylase of *Ms. trichosporium* OB3b is very similar to that of *Mc. capsulatus* (Bath) although differences exist in the distances between the iron atoms and the exogenous bridging ligands (Elango *et al.*, 1997). The mechanism

of methane oxidation in light of the X-ray crystal structures is discussed in Section 1.5.3.

1.5.1.2 Protein B

Protein B was first purified from *Mc. capsulatus* (Bath) by Green and Dalton (1985). The protein comprises a single 16 kDa subunit that has been observed to have a molecular mass of about 31 kDa by gel filtration, suggesting that it can exist as a dimer (H. Dalton, personal communication). Protein B is devoid of metals or cofactors and is a powerful regulator of sMMO activity, converting the enzyme from an oxidase to an oxygenase or *vice versa*, possibly in response to environmental conditions (Green and Dalton, 1985). In the absence of substrate, uncoupling occurs whereby NADH is oxidised and the hydroxylase and reductase catalyse the four electron reduction of molecular oxygen to water. In the presence of protein B, recoupling occurred and sMMO was switched from an oxidase to an oxygenase and so the reduction of oxygen to water no longer occurred. In the absence of substrate, electron transfer between the hydroxylase and the reductase did not occur, whilst the addition of substrate restored electron transfer and the substrate was oxidised. Therefore, in the presence of protein B, electrons are readily transferred to the hydroxylase when methane is present but shut down when methane is absent (Dalton, 1992). Green and Dalton (1985) found that protein B was susceptible to proteases and they postulated that this was associated with its role as a regulatory protein.

There appear to be subtle differences in the function of protein B in *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b. Fox *et al.* (1989) have shown that pure protein B from *Ms. trichosporium* OB3b is not required for electron transfer

between the hydroxylase and reductase or oxidation of substrate. In addition, the four electron reduction of oxygen to water does not occur. However, protein B increases the rate of reaction by about 150-fold and the protein was shown to comprise a homodimer of two 16 kDa subunits.

Protein B has been shown to bind to the α subunit of the hydroxylase in *Ms. trichosporium* OB3b (Fox *et al.*, 1991). In addition, EPR suggested that binding of protein B to the mixed valent hydroxylase caused a major alteration to the environment of the di-iron centre. A similar observation has been made for the *Mc. capsulatus* (Bath) proteins (DeWitt *et al.*, 1995). X-ray crystal structure data of the hydroxylase from *Mc. capsulatus* (Bath) has suggested that protein B could bind at the canyon formed between the αβ pairs (Rosenzweig *et al.*, 1993). Since the di-iron centre is exposed in this canyon then protein B could modulate the co-ordination of the di-iron centre through interactions with these helices. In particular, Rosenzweig *et al.* (1993) have suggested that the iron ligands Glu209 and Glu243 are probably affected. Protein B also has the ability to form a complex with the hydroxylase when it is in the fully reduced state, which was an important observation since this is the state responsible for binding of dioxygen (Fox *et al.*, 1989; Froland *et al.*, 1992). Protein B also caused a decrease in the redox potential of the hydroxylase in *Ms. trichosporium* OB3b (Paulsen *et al.*, 1994) and *Mc. capsulatus* (Bath) (Liu and Lippard, 1991; Kazlauskaite *et al.*, 1996) possibly due to a change in iron ligation brought about by a conformational change in the hydroxylase. Recently, radiolytic one-electron reduction at 77 K and EPR have been used to show that protein B has an effect on the hydroxylase di-iron site of *Ms. trichosporium* OB3b in the oxidised state (Davydov *et al.*, 1997). It was postulated that the complex of the di-ferric

hydroxylase with protein B may cause structural changes that could result in weakening of the proposed diamond core structure, to one with more readily dissociable bridging oxygen ligands. This could facilitate reaction with oxygen, following reduction of the hydroxylase.

During the cloning and DNA sequencing of the gene encoding protein B from *Mc. capsulatus* (Bath), a truncated form of protein B was identified, designated protein B' (Pilkington *et al.*, 1990). Results suggested that protein B' was a specific truncate of protein B possibly due to a modification at the C-terminus. These findings were consistent with the sensitivity of protein B to proteases (Green and Dalton, 1985). Protein B' has been characterised by Bhambra (1996) and further details of the inactivation of protein B to protein B' are described in Chapter 6.

1.5.1.3 Reductase

The reductase component of sMMO has been purified and extensively characterised from *Mc. capsulatus* (Bath) (Colby and Dalton, 1978; Colby and Dalton, 1979; Lund and Dalton, 1985; Lund *et al.*, 1985; Pilkington and Dalton, 1990) and *Ms. trichosporium* OB3b (Fox *et al.*, 1989; Fox *et al.*, 1991; Paulsen *et al.*, 1994; Liu *et al.*, 1997). The reductase from *Mc. capsulatus* (Bath) is a single subunit protein of 38.5 kDa that contains one FAD and one Fe_2S_2 centre per molecule (Colby and Dalton, 1979). The presence of a Fe_2S_2 centre was confirmed by EPR and NADH was postulated to be the natural electron donor. Copper ions inhibit the reductase by causing the loss of the Fe_2S_2 centre, thus preventing the transfer of electrons from the reductase to the hydroxylase (Green *et al.*, 1985). In addition, FAD is lost preventing the protein from accepting electrons from NADH. The reductase from *Ms.*

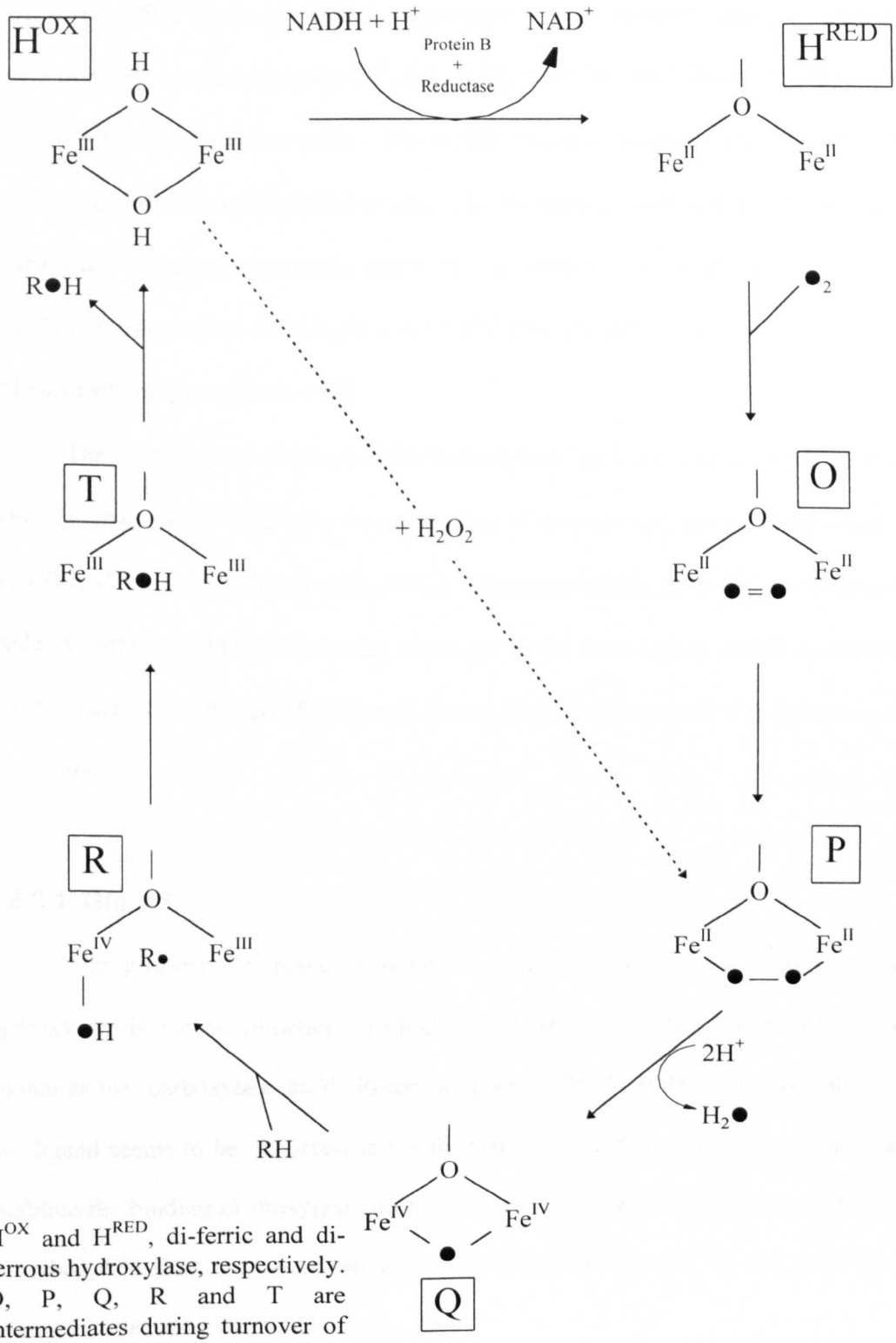
trichosporium OB3b is a single subunit protein of 40 kDa that contains the same FAD, Fe and S content as the reductase from *Mc. capsulatus* (Bath) (Fox *et al.*, 1991). The reductase of *Mc. capsulatus* (Bath) is readily reduced by NADH and electrons are transferred to the FAD centre which is fully reduced by the addition of two electrons (Lund and Dalton, 1985). Electrons are then transferred to the Fe_2S_2 centre of the reductase and then to the hydroxylase (Lund *et al.*, 1985). Since the reductase is present in only 10 % of the molar concentration of the other sMMO components, this may mean that the rate of electron transfer of the reductase is much higher than the hydroxylation rate for the hydroxylase (Fox *et al.*, 1991). It was therefore suggested that the lower reductase concentrations prevent the formation of reactive oxygen species.

The reductase component of sMMO from *Ms. trichosporium* OB3b has been shown to bind to the β subunit of the hydroxylase (Fox *et al.*, 1991) and in addition to its role as a supplier of electrons, recent evidence suggests that the reductase could also have a regulatory function since: (1) it causes a shift in product distribution (Froland *et al.*, 1992); (2) the reductase can bind to the hydroxylase at different sites and with different affinities (Fox *et al.*, 1991); (3) the redox potential of the hydroxylase is altered by the reductase (Paulsen *et al.*, 1994; Liu *et al.*, 1997).

1.5.2 Mechanism of sMMO

It has taken more than two decades of intense research to understand the mechanism of sMMO and although there are still questions to be answered, much is now known. An outline of the mechanism of sMMO is shown in Figure 1.5. All stages of the catalytic cycle intimately involve the di-iron centre and the initial step is

Figure 1.5: Proposed mechanism of sMMO. Adapted from Elango *et al.* (1997).



H^{OX} and H^{RED}, di-ferric and di-ferrous hydroxylase, respectively. O, P, Q, R and T are intermediates during turnover of the enzyme.

the reduction of the di-iron cluster to the di-ferrous Fe(II) state (Fox *et al.*, 1989). Oxygen then binds to the cluster in two steps, leading to the formation of compound P (Lee *et al.*, 1993; Liu *et al.*, 1995). This species spontaneously converts to a high valent iron-oxo species (compound Q) (Lee *et al.*, 1993) in which both irons appear to be in the Fe(IV) oxidation state. The highly oxidised di-iron cluster reacts with substrate to form the hydroxylated product and the resting di-ferric (FeIII) state of the hydroxylase. Hydrogen peroxide can replace protein B, the reductase, oxygen and NADH in the activation of the hydroxylase of sMMO during catalysis of the oxidation of hydrocarbons (Jiang *et al.*, 1993).

The X-ray crystal structure of the hydroxylase has been used to identify amino acids that may have key roles in the mechanism of methane oxidation (Rosenzweig *et al.*, 1993, 1995, 1996; Elango *et al.*, 1997). The most striking observations have been made by comparing the X-ray crystal structures of the hydroxylase of *Mc. capsulatus* (Bath) in the oxidised (FeIII-FeIII) and reduced (FeII-FeII) state at 1.7 Å (Rosenzweig *et al.*, 1995).

1.5.2.1 Glu243

The greatest difference between the oxidised and reduced states of the hydroxylase is the coordination of Glu243 with respect to the di-iron (II) centre, known as the 'carboxylate shift' (Rosenzweig *et al.*, 1995, 1996). The flexibility of this ligand seems to be important in the formation of the di-iron (II) centre and may facilitate the binding of dioxygen and formation of the high valent iron-oxo species. A carboxylate shift has also been identified upon oxidation of the R2 subunit of ribonucleotide reductase (Stubbe *et al.*, 1990).

1.5.2.2 Thr213 and Cys151

Thr213 and Cys151 are the only protonated amino acids in the active site and have therefore been implicated as having key roles as proton donors in the hydroxylation mechanism. Site-directed mutagenesis of the analogous residue to Thr213 in ribonucleotide reductase (designated Thr252) caused uncoupling (Raag *et al.*, 1991). This confirmed its proposed role as a supplier of electrons for the formation of intermediates and products during the catalytic cycle. In addition, Thr213 is located on the same helix as the proposed binding site for protein B (Rosenzweig *et al.*, 1993). Changes in the active site structure could be brought about following the binding of protein B, in such a way that the hydrogen bonding network involving Thr213 would be disrupted (Rosenzweig *et al.*, 1995). Protein B may therefore serve in fine tuning the redox potential of the di-iron centre, a role for protein B that has been well documented in previous studies (Liu and Lippard, 1991; Paulsen *et al.*, 1994; Kazlauskaite *et al.*, 1996).

Cys151 occupies the same position as the functionally important tyrosyl radical in the R2 protein of ribonucleotide reductase. This suggests that Cys151 may also be important in regulating the redox potential of the di-iron centre (Rosenzweig *et al.*, 1993). However, recent evidence suggests that Cys151 may not be critical to catalytic function (Elango *et al.*, 1997). By comparing the amino acid sequence of the hydroxylase with the di-iron containing enzymes toluene-4-monooxygenase, phenol hydroxylase and stearoyl-ACP desaturase, the residue equivalent to amino acid 213 is always Thr (Fox *et al.*, 1994). In contrast, the homologous protein to Cys151 of sMMO and Tyr122 of ribonucleotide reductase in toluene-4-monooxygenase and

stearoyl-ACP desaturase is Glu or Leu respectively, suggesting that this amino acid is not conserved.

1.5.2.3 Phe188 and Phe192

With the exception of Thr213 and Cys151, all of the amino acids at the active site are hydrophobic. In particular, Phe188 and Phe192 could clamp methane and oxygen into place (Rosenzweig *et al.*, 1993). Subsequently, it has been proposed that these residues form part of the unique methane binding site of sMMO (George *et al.*, 1996) which is located less than 3 Å from the di-iron centre.

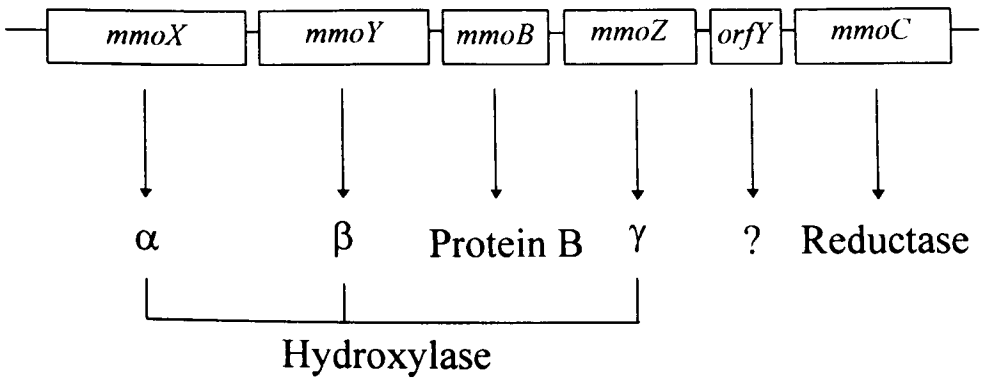
To understand precisely the roles of these amino acids, site-directed mutagenesis must be used to establish the effects of the active site mutations on the sMMO complex. This requires that the genes encoding sMMO are functionally expressed in a heterologous host which to date has been unsuccessful (Section 1.13). This has represented one of the major limitations in the further detailed study of the mechanism of sMMO.

1.6 Molecular biology of sMMO

Purified sMMO from the type X methanotroph, *Mc. capsulatus* (Bath) was used to generate N-terminal amino acid sequence information to identify and clone the sMMO gene cluster (Stainthorpe *et al.*, 1989, 1990). sMMO gene probes were then used to clone the sMMO operon from the type II methanotrophs, *Ms. trichosporium* OB3b (Cardy *et al.*, 1991a,b) and *Methylocystis* sp. strain M (McDonald *et al.*, 1997). In all three methanotrophs, DNA sequencing of the sMMO operon has revealed that the genes encoding the α, β and γ subunits of the hydroxylase (*mmoX, mmoY* and

mmoZ, respectively), protein B (*mmoB*) and the reductase (*mmoC*) are all linked on the chromosome (Figure 1.6). The function of *orfY* has remained elusive (Murrell,

Figure 1.6: The arrangement of the sMMO operon from *Mc. capsulatus* (Bath), *Ms. trichosporium* OB3b and *Mcy.* sp. strain M.



1992, 1994), however, ribosome binding sites are found upstream of *orfY* in all three sMMO operons supporting the validity of this open reading frame (McDonald *et al.*, 1997). In an attempt to elucidate its role (if any) in methane oxidation, *orfY* from *Mc. capsulatus* (Bath) has been purified and characterised from *E. coli*, as described in Chapter 5.

Comparison of the deduced amino acid sequences of the sMMO proteins of *Mc. capsulatus* (Bath), *Ms. trichosporium* OB3b and *Mcy.* sp. strain M (Table 1.2) demonstrates that sMMO from *Mcy.* sp. strain M is most closely related to sMMO from *Ms. trichosporium* OB3b (McDonald *et al.*, 1997). In addition, the α subunit of the hydroxylase exhibited the highest sequence similarity (88.9 to 97.3 %) and identity (80.2 to 95.4 %) amongst the three methanotrophs. The Glu-X-X-His

Table 1.2: Comparison of deduced amino acid sequences of the sMMO proteins of *Mcy.* sp. strain M, *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath). From McDonald *et al.* (1997).

Sequence compared	Compared organisms	% DNA sequence identity	% amino acid sequence identity	% amino acid sequence similarity
mmoX	*M. caps* and *M.* stM	76.9	82.3	89.9
	M. tric. and *M.* stM	91.4	95.4	97.3
	M. tric. and *M. caps.*	76.0	80.2	88.9
mmoY	*M. caps* and *M.* stM	67.9	61.0	76.2
	M. tric. and *M.* stM	91.0	88.3	92.4
	M. tric. and *M. caps.*	66.7	57.1	72.6
mmoZ	*M. caps* and *M.* stM	64.8	51.8	68.5
	M. tric. and *M.* stM	86.9	87.0	94.7
	M. tric. and *M. caps.*	63.8	50.0	67.9
mmoB	*M. caps* and *M.* stM	71.9	66.0	80.4
	M. tric. and *M.* stM	94.7	96.4	100
	M. tric. and *M. caps.*	71.1	66.0	81.1
orfY	*M. caps* and *M.* stM	64.5	42.9	65.5
	M. tric. and *M.* stM	79.2	59.8	72.5
	M. tric. and *M. caps.*	64.4	40.0	55.0
mmoC	*M. caps* and *M.* stM	62.8	51.3	71.0
	M. tric. and *M.* stM	80.8	80.5	86.4
	M. tric. and *M. caps.*	61.1	48.7	67.6

M. caps., *Mc. capsulatus* (Bath); *M. tric.*, *Ms. trichosporium* OB3b; *M.* stM, *Mcy.* sp. strain M.

sequence motif that coordinates the di-iron centre in the α subunit of the hydroxylase (discussed in Section 1.5.1.1) is also present in *Mcy.* sp. strain M (McDonald *et al.*, 1997) which further confirms the proposed active site structure of sMMO.

The reductase from *Mc. capsulatus* (Bath) (Stainthorpe *et al.*, 1990), *Ms. trichosporium* OB3b (Cardy *et al.*, 1991b) and *Mcy.* sp. strain M (McDonald *et al.*, 1997) exhibit significant homologies with ferredoxins from plants and bacteria and contain four conserved cysteine residues which are a common features of Fe_2S_2 centres of ferredoxins. This supported the biochemical evidence for a ferrodoxin-like Fe_2S_2 centre (Lund and Dalton, 1985; Fox *et al.*, 1989).

The deduced amino acid sequences of protein B show limited homologies with equivalent counterparts from other monooxygenases. Examples include: protein B from *Ms. trichosporium* OB3b which has a 46.7 % amino acid similarity and 27.8 % amino sequence identity with TbmC of toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150 (Johnson and Olsen, 1995); the P2 component of phenol hydroxylase from *Pseudomonas* sp. CF600 (Qian *et al.*, 1997) and the coupling protein of alkene monooxygenase of *Nocardia corallina* B276 (Saeki and Furuhashi, 1994). The β and γ subunits of the sMMO hydroxylase do not have any significant homologies with any other protein sequences (Murrell, 1992). Comprehensive reviews on the molecular biology of sMMO are available (Murrell 1992,1994).

1.7 Molecular ecology of methanotrophs

The number of methanotrophs isolated from environmental samples only represents a small fraction of the viable population of cells recovered, which may

reflect the conditions used for enrichment and isolation (Hanson and Hanson, 1996). Consequently, molecular ecological techniques have been used to identify methanotrophs present in different environments. This has been especially crucial since organisms that cannot be cultured can be detected using nucleic acid probes or by sequencing genes, amplified by PCR directly from environmental samples. Such a method has been successfully used to detect methanotrophs containing sMMO and pMMO genes in soil (McDonald *et al.*, 1995), peat bogs (McDonald *et al.*, 1996) and sea water (Holmes *et al.*, 1996).

An area of intense study at present is to isolate methanotrophs capable of oxidising atmospheric concentrations of methane. This work has arisen because pure cultures of methanotrophs do not show kinetic properties that explain methane oxidation at the low methane concentrations found in soils, in which the atmosphere is the primary source of methane (Hanson and Hanson, 1996). In a limited survey, of *Ms. trichosporium* OB3b, *Mm. rubrum*, *Mm. album* BG8 and *Mc. capsulatus* (Bath), only *Mc. capsulatus* (Bath) could consume atmospheric concentrations of methane (King, 1992). This could suggest that atmospheric methane oxidisers contain methane oxidation systems with different kinetic properties from those already characterised. Molecular ecology techniques will be crucial for the characterisation of these organisms that have not yet been isolated using standard laboratory techniques.

1.8 Comparison of pMMO and sMMO

Methanotrophs have higher growth yields on methane when expressing pMMO rather than sMMO (Leak and Dalton, 1986a,b). This has been attributed to the requirement of sMMO for NADH + H^+ as an electron donor whilst the pMMO

may use a higher potential electron donor, possibly cytochrome *b*-559/569 (Zahn and DiSpirito, 1996). pMMO has a higher affinity for methane than the sMMO (Hanson and Hanson, 1996). In addition, the inhibitor profile of sMMO differs to that of pMMO (Scott *et al.*, 1981b; Stanley *et al.*, 1983) owing to the insensitivity of pMMO to potassium cyanide, 2-mercaptoethanol, thiourea, 8-hydroxyquinoline and ethyne, all of which cause greater than 29 % inhibition to sMMO. The substrate specificities of sMMO and pMMO also differ. sMMO is capable of oxidising more than 250 different hydrocarbons compared to pMMO which has a much narrower substrate specificity and is only capable of oxidising C5 or smaller hydrocarbons (Burrows *et al.*, 1984; Lipscomb, 1994). The reason for the production of sMMO by some methanotrophs is unclear. Hanson and Hanson (1996) have proposed that it may be a survival mechanism in environments where copper limits the growth of methanotrophs that can only synthesise pMMO.

1.9 Transcriptional regulation of MMO expression

The switch between expression of pMMO and sMMO in methanotrophs that contain both MMO operons has been an area of great interest. The type of enzyme expressed is dependent upon the availability of copper ions in the growth medium; pMMO activity is observed at a high copper-to-biomass ratio whilst organisms grown under conditions of low copper-to-biomass ratios express sMMO (Stanley *et al.*, 1983). The physiological growth conditions under which *Ms. trichosporium* OB3b switches between using pMMO and sMMO have been well defined (Burrows *et al.*, 1984; Tsien *et al.*, 1989; Park *et al.*, 1991; Park *et al.*, 1992; Martin, 1994) and generally, sMMO is expressed when the concentration of copper ions in the growth

medium is less than 0.25 μM. An exception to this is found in heat tolerant methanotrophs (Bodrossy *et al.*, 1995) which express sMMO at 0.8 μM copper, presenting an interesting phenomenon worthy of further investigation.

Detailed studies by Nielsen *et al.* (1996, 1997) using Northern blot and primer extension analysis have provided much information on the transcriptional regulation of MMO genes. Growth of *Ms. trichosporium* OB3b under conditions of low copper-to-biomass ratios identified three sMMO-specific mRNAs that encoded (1) the *mmoX* gene product alone; (2) the *mmoY, mmoB, mmoZ, orfY* and *mmoC* gene products; (3) the *mmoY, mmoB* and *mmoZ* gene products. Primer extension analysis of the region just upstream of *mmoX* located the 5' end of mRNA 1 to G_{424} which is 147 nucleotides upstream of the start codon. Preceding this position are putative promoters similar to those recognised by *E. coli* RNA polymerase containing σ^{70} or σ^{54} (Cardy *et al.*, 1991a,b; Murrell, 1994). Of the two putative promoter sequences, the σ^{54} promoter had the closest homology to the *E. coli* consensus sequence and was located at an optimal position with respect to transcriptional initiation at G_{424}.

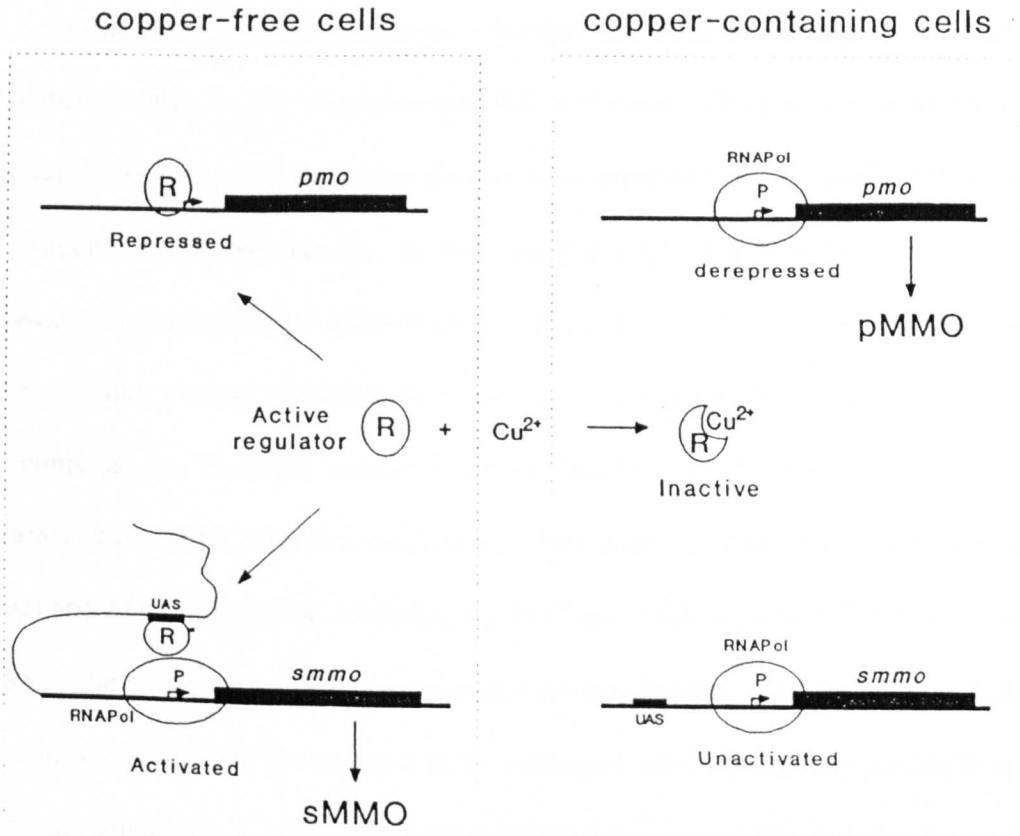
Nielsen *et al.* (1996) have also shown that *Mc. capsulatus* (Bath) grown at a low copper-to-biomass ratio yielded three sMMO-specific transcripts (1) *mmoX* only; (2) *mmoX, mmoY, mmoB* and *mmoZ*; (3) the whole sMMO operon. Primer extension analysis was used to show that transcription was initiated 87 nucleotides upstream of the *mmoX* start codon from a single promoter similar to those recognised by *E. coli* RNA polymerase containing σ^{70}.

To determine the effect of copper upon the transcription of the sMMO operon, 50 μM copper sulphate was added to chemostats of *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath) expressing sMMO (Nielsen *et al.*, 1996, 1997). sMMO and

pMMO-specific riboprobes were then used to follow the transcription of the MMO operons after the addition of copper. In *Mc. capsulatus* (Bath) the level of sMMO-specific mRNAs decreased in response to the sudden availability of copper ions when all other growth parameters were kept constant (Nielsen *et al.*, 1996). A similar observation was made for *Ms. trichosporium* OB3b (Nielsen *et al.*, 1997). These studies have shown that a specific inhibition of the sMMO promoter was brought about by the sudden availability of copper ions. However, copper-dependent changes in the stability of sMMO mRNAs cannot be ruled out.

Riboprobes specific to the *pmo* genes from *Mc. capsulatus* (Bath) hybridised to a 3.3 kb *pmoCAB* mRNA which was induced when cells expressing sMMO were switched by the addition of copper to express pMMO (Nielsen *et al.*, 1997). A 4 kb *pmoA* specific mRNA was also observed in *Ms. trichosporium* OB3b when grown under the same growth conditions. Based upon these studies, Nielsen *et al.* (1997) have proposed the first hypothetical model for the transcriptional regulation of MMO genes (Figure 1.7). Expression of sMMO genes could be subject to positive control such that copper ions would regulate sMMO expression by inactivation of an activator protein. In copper-free cells, a regulator would bind to the pMMO operator and repress transcription of these genes. The same (or another) regulator could bind to an activator sequence upstream of the sMMO promoter and bring about transcription of the sMMO genes. In copper-excess cells, binding of copper ions to the regulator could induce a conformational change such that it would no longer bind to the pMMO promoter and so the pMMO genes would be transcribed whilst transcription of the sMMO genes was repressed.

Figure 1.7: Hypothetical model for the copper-dependent transcriptional regulation of pMMO and sMMO genes. Reproduced from Nielsen *et al.* (1997).



1.10 Gene transfer

A major problem in the development of genetic systems for methanotrophs has been the lack of a suitable transformation method and a way of maintaining heterologous DNA in these organisms (Murrell, 1994). This problem has been partly overcome by the use of plasmids that have a broad host range spanning many Gram negative bacteria (discussed in Section 7.1). A number of large plasmids have been identified in methanotrophs ranging from 55 to 190 kb in *Mm.*, *Mcy.* and *Ms.* (Lidstrom and Wopat, 1984). However, difficulties in the large scale isolation of

plasmid DNA from these organisms has restricted their development as cloning vectors (Section 7.1).

Little success has been achieved with electroporation of *Mc. capsulatus* (Bath) (Davidson, 1991) or *Ms. trichosporium* OB3b (Martin, 1994) as a transformation method. It was proposed that either the electrical impulses were of insufficient power to generate membrane damage, or that the presence of extracellular protein or polysaccharide reduced the effectiveness of the electric pulse (Martin, 1994). The most effective method of transforming methanotrophs with plasmid DNA has been *via* conjugation. Conjugal transfer has been reported for *Ms. trichosporium* OB3b (Warner *et al.*, 1980; Al-Taho and Warner, 1987; Martin, 1994; Martin and Murrell, 1995) and *Mm. album* BG8, *Methylocystis* POC and *Methylosinus* 6 (Lidstrom *et al.*, 1984). The method involved a tri-parental mating technique using two *E. coli* donor strains, one containing the plasmid to be mobilised into the recipient methanotroph and one containing a helper plasmid (pRK2013) to supply the transfer functions needed for plasmid mobilisation. A detailed study of gene transfer in *Ms. trichosporium* OB3b by Martin (1994) using tri-parental matings resulted in the isolation of transconjugant methanotrophs at an isolation frequency of 10^{-8} to 10^{-9} for the broad host range plasmids pDSK509 (Keen *et al.*, 1988) and pVK100 (Knauf and Nester, 1982). However, problems with reproducibility were encountered. Consequently the technique was modified by using *E. coli* S17-1 (Simon *et al.*, 1983) which carries the RP4 transfer functions integrated into the genome, resulting in highly efficient conjugal transfer of plasmids carrying the RP4 *mob* sequence. Optimisation of the filter mating conditions (Martin, 1994) led to consistent transformation frequencies of 10^{-7} to 10^{-8} for pDSK509 and pVK100. This

transformation technique has been used successfully to transfer plasmids from *E. coli* S17-1 to *Mm. album* BG8, *Mcy. parvus* OBBP and *Ms. trichosporium* OB3b (Martin, 1994; Martin and Murrell, 1995; Finch, 1997) and was used throughout this study (Chapters 7 and 8).

1.11 Mutant isolation

Using mutagens such as nitrosoguanidine, ethyl methane sulphonate and ultraviolet light indicated that it was difficult to increase the spontaneous mutation frequencies of methanotrophs to produce resistance to amino acid analogues and antibiotics (Harwood *et al.*, 1972). Greater success has been achieved using dichloromethane (DCM) which is cooxidised by MMO to the toxic product carbon monoxide which kills the cell (Dalton, 1980; Murrell, 1994). Therefore, methanotrophs grown on methanol agar plates that are DCM-resistant are potential MMO-minus mutants. Methanol-adapted *Ms. trichosporium* OB3b (Nicolaidis and Sargent, 1987) and *Mm. album* BG8 (McPheat *et al.*, 1987b) were incubated on methanol plates in a DCM atmosphere. This gave rise to DCM-resistant colonies at frequencies of about 10^{-4}. Some of these mutants could not grow on methane. However, the medium used by Nicolaidis and Sargent (1987) contained enough copper to repress transcription of the sMMO operon and so it was most likely that pMMO-minus mutants were selected and whether they could produce functional sMMO was not tested. *Mm. album* BG8 produces only pMMO and hence it was most likely that mutations had occurred within the pMMO structural genes in this organism at least (McPheat *et al.*, 1987b).

Subsequent studies have re-examined the use of DCM as a selective agent for the isolation of MMO-minus mutants, whilst paying greater attention to the growth conditions of the methanotrophs (Murrell, 1994). This has been performed in conjunction with a quick and reliable whole cell assay that can be used to detect for functional sMMO activity. The principle of the assay relies on the oxidation of naphthalene to naphthol by sMMO and not pMMO. The naphthols that are formed can be visualised with tetrazotised O-dianisidine (TOD) which forms a purple diazo dye (Brusseau *et al.*, 1990). In addition, the whole cell sMMO assay can be performed on plates and does not kill the cells and so they can be recovered by subculturing on to fresh agar plates under the appropriate growth conditions (Graham *et al.*, 1992). A study that used this combination of techniques was performed by Phelps *et al.* (1992) to isolate and characterise pMMO-minus mutants of *Ms. trichosporium* OB3b. To improve the frequency of isolation of pMMO-minus mutants, sMMO expression was suppressed by high concentrations of copper in the selection plates; excess methanol was included with each DCM addition to inhibit possible sMMO-mediated activation of DCM to carbon monoxide and addition of yeast extract was used to improve growth on methanol. Five stable mutants were isolated that were deficient in pMMO and also lacked intracellular stacked membranes which correlate with the expression of pMMO in wild-type *Ms. trichosporium* OB3b. Constitutive expression of sMMO was observed in the presence of elevated levels of copper (up to 5 μM) which would normally repress sMMO expression in the wild-type. Phelps *et al.* (1992) concluded that the loss of pMMO activity may be as a result of a defect in the pMMO structural genes or in a regulatory gene encoding a protein(s) responsible for copper uptake. Subsequent phenotypic characterisation of

the five mutants (Fitch *et al.*, 1993) suggested that they arose due to defects in copper uptake and metabolism rather than from changes in sMMO expression or enzyme stability. Berson and Lidstrom (1997) cloned and characterised a gene, *corA* encoding a 28.5 kDa copper-repressible protein from *Mm. album* BG8. CorA had homology with calcium release channel proteins suggesting that it may belong to a family of divalent metal membrane channels. Inactivation of the gene by the insertion of a kanamycin-resistance cassette, resulted in mutants that would not grow in liquid culture, suggesting that *corA* had a vital role for growth. Since copper uptake could not be characterised in the CorA mutant, its role in copper transport could not be elucidated, but provides some evidence for a copper uptake system in *Mm. album* BG8.

Isolation of DCM-resistant mutants of *Ms. trichosporium* OB3b was also attempted by Martin (1994) to generate sMMO-minus mutants for complementation analysis. Although organisms were grown in the presence of DCM under conditions of low copper (resulting in sMMO expression), an sMMO-plus, pMMO-minus phenotype was still obtained. Martin (1994) proposed that DCM may directly affect membrane structures and that a basal expression level of pMMO could have occurred in the absence of copper. In addition, the DCM-resistance was unstable, which was an observation made throughout the studies of using DCM as a selective agent (Murrell, 1994). An alternative strategy to isolate stable sMMO-minus mutants of *Ms. trichosporium* OB3b was achieved using marker exchange mutagenesis (Martin and Murrell, 1995) and is discussed in detail in Section 8.1.

1.12 Commercial applications of methanotrophs

The unique abilities of the MMOs of methanotrophic bacteria to catalyse reactions of environmental and commercial importance has culminated in the discovery that methanotrophs can degrade toxic pollutants. This may lead to their greatest potential for commercial application at present (Tsien *et al.*, 1989; Fox *et al.*, 1990; Oldenhuis and Janssen, 1993; Hanson and Hanson, 1996).

Synthetic chlorinated hydrocarbons are used as solvents in degreasing metals, in dry cleaning, as propellants, fumigants, anaesthetics and in the manufacture of plastics (Hanson and Brusseau, 1994). As a result of their widespread use, careless disposal and their chemical stability, they have become the most commonly detected groundwater pollutant in the United States and other countries (Ensley, 1991). Bacteria capable of utilising TCE as a sole carbon and energy source have not been discovered (Hanson, 1992), however, bacteria capable of cooxidising (and thus degrading) this compound have been discovered, including the MMOs of methanotrophs (Dalton, 1980; Oldenhuis *et al.*, 1989; Tsien *et al.*, 1989). *Ms. trichosporium* OB3b expressing sMMO has the highest rate of TCE degradation at >150 nmol min^{-1} mg $protein^{-1}$ in whole cells (Tsien *et al.*, 1989) and 680 nmol min^{-1} mg $protein^{-1}$ for pure sMMO (Fox *et al.*, 1990). TCE oxidation by whole cells of *Ms. trichosporium* OB3b is at least two orders of magnitude higher than cells of other bacteria expressing other enzymes including toluene-2-monooxygenase of *Pseudomonas cepacia* (Folsom *et al.*, 1990) and propane monooxygenase of *Mycobacterium* sp. (Wackett and Gibson, 1988). Different bioreactor configurations for the degradation of TCE and other halogenated hydrocarbons by *Ms. trichosporium* OB3b have been described (e.g. Phelps *et al.*, 1990; Alvarez-Cohen *et al.*, 1991).

However, a number of problems cause complications in attempts to use methanotrophs for bioremediation in bioreactor conditions including: (1) competition between methane and solvents for the active site of sMMO; (2) inactivation of the cooxidation capacity of cells by metabolites of halogenated compounds; (3) limited supply of reducing equivalents for the sMMO (Oldenhuis and Janssen, 1993; Hanson and Hanson, 1996); (4) inactivation of sMMO expression by copper.

In situ bioremediation has also been investigated (reviewed by Hanson and Hanson, 1996) and it has been shown that the supply of methane and air mixtures to river sites stimulates methanotrophic TCE degradation. In addition, Hanson *et al.* (1993) have observed TCE degradation (0.4 nmol g^{-1} day^{-1}) by plant material during the summer months due to methanotrophic bacteria that are associated with aquatic plants and contain sMMO.

There is also interest in methanotrophs for the production of single-cell protein (Leak, 1992), however, methanol has replaced methane as a potential feedstock in industrial fermentations involving methylotrophic bacteria (Lidstrom and Stirling, 1990). Methanol is abundant, its price is stable (and low), it is easily stored and many organisms can grow on methanol as a carbon and energy source. There is also interest in using the MMOs of methanotrophic bacteria for the production of chemicals with commercial value. For example, immobilised cells of *Ms. trichosporium* OB3b have been used for the biosynthesis of methanol from methane (Mehta *et al.*, 1991).

The isolation and characterisation of 14 heat-tolerant methanotrophs (Bodrossy *et al.*, 1995) has major implications for the biotechnological applications of methanotrophs. Aside from the fact that bioconversions could be performed at higher temperatures, more important is the observed copper-resistant sMMO activity of the

isolates. Environments where decontamination would be required such as groundwater and waste water for example, would not be copper free and so repression of sMMO transcription would normally occur.

1.13 Heterologous expression of sMMO

The expression of genes encoding sMMO in a heterologous host will allow site-directed mutagenesis studies to be performed on the enzyme. Amino acids have been identified within the hydroxylase component of sMMO that are likely to have an intrinsic role in the mechanism of methane oxidation (Section 1.5.3). Although a large number of biochemical techniques have been used to elucidate the mechanism of sMMO, the function of key active site residues cannot be truly elucidated until site-directed mutagenesis can be performed. Unfortunately, the heterologous expression of sMMO has been problematic and, to date, a reliable heterologous expression system has not been reported. Using the T7-RNA polymerase expression system (Studier and Moffatt, 1986), genes encoding sMMO from *Mc. capsulatus* (Bath) were over-expressed in *E. coli* (West *et al.*, 1992). Functional expression of protein B and the reductase was achieved, however, the hydroxylase was inactive, possibly due to the inability of *E. coli* to assemble the protein correctly. The heterologous expression of sMMO genes in *E. coli* is discussed further in Section 3.1. The heterologous expression of sMMO from *Ms. trichosporium* OB3b has been reported in *Pseudomonas putida* (Jahng and Wood, 1994; Jahng *et al.*, 1996) and *E. coli* (Jahng *et al.*, 1996). Although the sMMO genes from *Ms. trichosporium* OB3b were inactive in *E. coli* (Jahng *et al.*, 1996), functional expression was reported in *P. putida* F1 (Jahng and Wood, 1994). Heterologous expression of sMMO from *Ms. trichosporium* OB3b

is discussed in further detail in Section 4.1. In addition, Martin (1994) used broad host range plasmids to express the sMMO genes in methanotrophs that only express pMMO. This heterologous expression strategy is discussed in detail in Section 7.1. Finally, Martin (1994) and Finch (1997) have complemented sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b with plasmid-encoded genes, which is discussed in Section 8.1.

1.14 Project aims

A major problem in the molecular biology and protein engineering of sMMO has been the inability to functionally express the enzyme in a heterologous host. Successful expression of sMMO in an active form would clearly be a major breakthrough and would lead to the engineering of the enzyme, especially with respect to site-directed mutagenesis of key active site residues within the hydroxylase of sMMO. Therefore, the aims of the work presented in this thesis were:

1. To establish further why the sMMO genes were inactive in *E. coli* and to use a variety of different methods in an attempt to obtain active recombinant sMMO.

2. To investigate further the heterologous expression system reported by Jahng and Wood (1994), to obtain functional expression of sMMO from *Ms. trichosporium* OB3b in *P. putida*.

3. To determine the feasibility of (i) expressing sMMO genes in pMMO-only methanotrophs and (ii) complementing sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b with plasmid-encoded genes.

4. To use site-directed mutagenesis to establish the role of various amino acid residues in the mechanism of methane oxidation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals and biochemicals were of analytical grade from Aldrich Chemical Company Ltd., Dorset, UK; BDH Chemicals Ltd., Dorset, UK and Sigma Chemical Company Ltd., Dorset, UK. Restriction endonucleases and DNA modifying enzymes were obtained from Gibco BRL, Paisley, UK (unless otherwise stated). Methane/CO_2 and Nitrogen were obtained from Linde Gas Ltd., Stoke on Trent, UK.

2.2 Bacterial strains

2.2.1 *E. coli*

E. coli strain	Source
AD202	Nakano *et al.* (1994)
BL21(DE3)	Novagen, Abingdon, UK
BL21(DE3) pLysE	Novagen
BL21(DE3) pLysS	Novagen
DH1	Low (1986)
DH5α	Hanahan (1983)
INVαF'	Invitrogen BV, Leek, Netherlands
NM522 mutS	Pharmacia Biotech, Middlesex, UK
S17-1	Simon *et al.* (1983)
SG20252	Trisler and Gottesman (1984)
UT5600	McIntosh *et al.* (1979)

2.2.2 *P. putida*

P. putida NCIMB 11767, NCIMB 8859, NCIMB 10007 and NCIMB 8248 were obtained from the University of Warwick culture collection.

P. putida F1 (Wackett and Gibson, 1988) was provided by Dr. T. Wood (University of California, USA).

2.2.3 Methanotrophs

***Mcy. parvus* OBBP**	Type II methanotroph possessing pMMO only. University of Warwick culture collection.
***Mm. album* BG8**	Type I methanotroph possessing pMMO only. University of Warwick culture collection.
***Mc. capsulatus* (Bath)**	Type X methanotroph possessing pMMO and sMMO. University of Warwick culture collection.
***Ms. trichosporium* OB3b**	Type II methanotroph possess sMMO and pMMO. University of Warwick culture collection.
***Ms. trichosporium* OB3b mutant F**	sMMO minus marker exchange mutant strain F (Martin and Murrell, 1995).

2.2.4 Plasmids

Plasmid	Characteristics	Source
pVK100	Km^R Tc^RIncP1, Mob^+ conjugative BHR vector (25 kb)	Knauf and Nester (1982)
pVK100Sc	pVK100 containing the sMMO operon of *Ms. trichosporium* OB3b as a *Bam*HI fragment (35 kb)	Martin (1994)
pVK104	pVK100 containing the sMMO cluster of *Mc. capsulatus* (Bath) as an *Eco*RI fragment (35 kb)	DeMarco (1996)
pDSK509	Km^R Mob^+ conjugative BHR vector (9.1 kb)	Keen *et al.* (1988)
pDSK509Ω	pDSK509 containing a Sm^R cassette as a *Bam*HI fragment (11.1 kb)	Martin (1994)
pDSK509ΩSc	pDSK509Ω containing the sMMO operon of *Ms. trichosporium* OB3b as a *Kpn*I fragment (21 kb)	Martin (1994)
pCH4	Ap^R *Eco*RI fragment of *Mc. capsulatus* (Bath) DNA containing the sMMO operon, in pBR325 (15 kb)	Stainthorpe *et al.* (1990)
pT7-5	Ap^R expression vector containing the T7 RNA polymerase promoter (2.4 kb)	S. Tabor (1990)
pT7-5A	pT7-5 containing an *mmoX-mmoY* *Eco*RI/*Hin*dIII fragment and a *mmoZ* *Hin*dIII/*Bam*HI fragment subcloned from pCH4 (6 kb)	C. West, unpublished
pT7-5α	pT7-5 containing a *Bam*HI/*Pst*I fragment from pCH4 containing *mmoX* (4 kb)	C. West, unpublished
pEB-51	1.7 kb *mmoB Pst*I fragment of pCH4 subcloned into pT7-5 (4.4 kb)	West *et al.* (1992)
pEC-71	*Nde*I/*Bam*HI PCR fragment containing the whole of *mmoC*, in pT7-5 (4.8 kb)	West *et al.* (1992)
pT7-5orfY	*Pst*I fragment subcloned from pCH4 containing *orfY*, in pT7-5 (3 kb)	C. West, unpublished

Plasmid	Characteristics	Source
pT7-5sMMO	pT7-5 containing a *Bam*HI fragment from pCH4 containing the sMMO operon from *Mc. capsulatus* (Bath) (12 kb)	C. West, unpublished
pT-Trx	Cm^R T7 expression vector containing the *E. coli TrxA*-coding region	Yasukawa *et al.* (1995)
pSMMO20	Ap^R and Cm^R BHR plasmid containing the sMMO operon of *Ms. trichosporium* OB3b as a *Sma*I fragment (15 kb)	Jahng and Wood (1994)
pSMMO40	pSMMO20 containing the *hok/sok* locus as a *Bam*HI fragment (16 kb)	Jahng *et al.* (1996)
pGEX-2T	Ap^R translational fusion expression vector containing the *Shistosoma japonicum* GST gene under the control of the *tac* promoter (5 kb)	Pharmacia Biotech
pGEX-orfY	pGEX-2T containing *orfY* from *Mc. capsulatus* (Bath) as a *Bam*HI/*Eco*RI fragment (5.5 kb)	C. West, unpublished
pGEX-WTB	pGEX-2T containing *mmoB* from *Mc. capsulatus* (Bath) as a *Bam*HI/*Eco*RI fragment (5.5 kb)	This study
pGEX-G13Q	pGEX-WTB containing a Gly13 to Gln13 mutation in *mmoB*	This study
pGEX-MTB	pGEX-2T containing *mmoB* from *Ms. trichosporium* OB3b as a *Bam*HI/*Eco*RI fragment (5.5 kb)	S. Harris, unpublished
pGEX-MMOC	pGEX-2T containing *mmoC* from *Mc. capsulatus* (Bath) as a *Eco*RI/*Bam*HI fragment (6 kb)	This study
pGEX-MTC	pGEX-2T containing *mmoC* from *Ms. trichosporium* OB3b as a *Bam*HI/*Sma*I fragment	S. Harris, unpublished
pJB3Km1	Ap^R RK2 BHR vector (6 kb)	Blatny *et al.* (1997)
pJL-sMMO	pJB3Km1 containing the sMMO operon from *Mc. capsulatus* (Bath) as an *Eco*RI fragment (18 kb)	This study

2.3 Growth media for micro-organisms

Media were made with distilled water and sterilised by autoclaving at 120 °C for 15 min. Water soluble, heat labile solutions were sterilised using 0.2 μm pore-size disposable sterile filter units (Millipore, Watford, UK). For solutions dissolved in solvents, 0.12 μm Millex-FG hydrophobic solvent resistant filters were used (Millipore).

2.3.1 *E. coli*

Luria Bertani medium (LB) or Terrific broth, as detailed in Sambrook *et al.* (1989), were routinely used for culturing *E. coli*. For long term storage, sterile glycerol (500 μl) was added to 500 μl of fresh overnight cultures of *E. coli*, mixed well by vortexing and then frozen in liquid nitrogen for storage at -70 °C. To reculture the *E. coli* strain, 50 μl of the glycerol stock were used to inoculate 10 ml of LB.

E. coli strains were also grown in M9 minimal media, pH 7.6 (Jahng and Wood, 1994):

Solution A	
Na_2HPO_4	7 g litre^{-1}
KH_2PO_4	3 g litre^{-1}
NaCl	5 g litre^{-1}
NH_4Cl	1 g litre^{-1}

Solution B	
Glucose	5 g litre^{-1}

$MgSO_4.7H_2O$	0.25 g litre^{-1}
$CaCl_2.2H_2O$	0.02 g litre^{-1}

Solutions A and B were autoclaved separately and mixed at a ratio of 4:1.

E. coli strains were also grown in optimised Whittenbury media, pH 7.0 (Jahng *et al.*, 1996):

Glucose	10 g litre^{-1}
NH_4Cl	1 g litre^{-1}
$MgSO_4.7H_2O$	1 g litre^{-1}
$CaCl_2.2H_2O$	0.2 g litre^{-1}
Na_2HPO_4	0.86 g litre^{-1}
KH_2PO_4	0.53 g litre^{-1}
Na_2MoO_4	0.02 mg litre^{-1}
$MnCl_2.4H_2O$	0.02 mg litre^{-1}
H_3BO_3	0.01 mg litre^{-1}
$CoCl_2.6H_2O$	0.05 mg litre^{-1}
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.41 mg litre^{-1}
FeNaEDTA	0.25 mg litre^{-1}

For preparation of solid media, 2 % (w/v) Bacto-agar (Difco, Michigan, USA) was added prior to sterilisation.

2.3.2 *P. putida*

LB (Sambrook *et al.*, 1989), M9 and optimised Whittenbury media (described in Section 2.3.1) were used to culture *P. putida*. For the growth of *P. putida* using toluene as a sole source of carbon and energy, 1 ml of toluene was added to the centre well of a 250 ml Quick fit flask, containing 50 ml of glucose-free M9 medium. Glucose-free M9 medium was prepared as described in Section 2.3.1, except that glucose was omitted. Long term storage of *P. putida* strains was as described in Section 2.3.1.

2.3.3 Methanotrophs

Methanotrophs were routinely grown in Nitrate Minimal Salts (NMS) media:

$MgSO_4$	1 g litre^{-1}
KNO_3	1 g litre^{-1}
$CaCl_2.2H_2O$	0.2 g litre^{-1}
Trace element solution	1ml litre^{-1}
$NaMoO_4.2H_2O$	0.5 mg litre^{-1}
FeNaEDTA	3.8 mg litre^{-1}

Trace element solution contained:

$FeSO_4.5H_2O$	0.5 g litre^{-1}
$ZnSO_4.7H_2O$	0.4 g litre^{-1}
H_3BO_3	0.015 g litre^{-1}
$CoCl_3.6H_2O$	0.05 g litre^{-1}
Na_2EDTA	0.25 g litre^{-1}

$MnCl_2.4H_2O$	0.02 g litre^{-1}
$NiCl_2.6H_2O$	0.01 g litre^{-1}
$CuSO_4.5H_2O$	0.2 g litre^{-1}

1 ml of sterile phosphate buffer was added to each 99 ml of sterile 1 × NMS when cool. 100 × phosphate buffer (pH 6.8) contained KH_2PO_4, 26 g litre^{-1}; $NaHPO_4.12H_2O$, 71.6 g litre^{-1}. For preparation of solid media, 2 % (w/v) Bacto-agar (Difco) was added to the NMS (minus phosphates) prior to sterilisation.

Copper-free 0.1 × NMS used for sMMO studies was formulated in the same way as for NMS except that $CuSO_4.5H_2O$ was omitted from the trace element solution and the medium was used at 0.1 × concentration. Copper contamination was removed from water and glassware according to the methods of Tsien *et al.* (1989). Chelex 100 ion exchange resin was obtained from Bio-Rad Labs, Watford, UK.

2.4 Culturing conditions

2.4.1 *E. coli*

E. coli strains were routinely grown in 10 ml batch cultures at 37 °C with shaking at 200 rpm. For growth of *E. coli* in copper-free conditions, M9 and optimised Whittenbury medium both made using copper-free water and glassware as described in Section 2.3.3 were used. Antibiotics appropriate to the *E. coli* strain and plasmid were included in the growth medium to ensure plasmid maintenance and strain purity. *E. coli* was also grown in 50 ml and 1 litre volumes under identical conditions. 16 litre cultures of *E. coli* were grown at 37 °C in a 20 litre fermenter (L. H. Engineering, Stoke Poges, UK) with an agitation speed of 450 rpm, and an air flow

rate of 50-500 ml min^{-1}. pH was maintained at 7.0 *via* the automatic addition of 1 M HCl or 1 M NaOH.

2.4.2 *P. putida*

P. putida was cultured in the same way as described for *E. coli* except that 10 ml, 50 ml and 1 litre shake flasks were incubated at 30 °C. Copper-free growth of *P. putida* was achieved using M9 and optimised Whittenbury medium made in copper-free water and glassware as described in Section 2.3.3. Large scale cultures of *P. putida* were grown at 30 °C in a 20 litre chemostat (L. H. Engineering) with an agitation speed of 550 rpm, and an air flow rate of 50-500 ml min^{-1}. pH was maintained at 7.0 *via* the automatic addition of 0.5 M HCl or 0.5 M NaOH.

2.4.3 Methanotrophs

Methanotroph batch cultures were grown in 250 ml Quickfit flasks sealed with rubber Suba Seals (W. H. Freeman, Barnsley, UK). Cultures were gassed by removing 50 ml of air from the flask and injecting 60 ml of a methane/CO_2 mixture (95 % and 5 % (v/v) respectively). Cultures containing 1 × NMS were referred to as 'copper-plus', whilst those made with copper-free 0.1 × NMS were referred to as 'copper-free'. Cultures were incubated at 30 °C on an orbital shaker at 200 rpm and required about 5 days to reach a stationary phase OD_{540} of approximately 0.8.

Plate cultures were grown on NMS agar media in plastic Tupperware™ boxes gassed with methane and CO_2 mixtures (95:5) and were incubated at 30 °C. Agar plates containing 1 × NMS were referred to as 'copper-plus' and those made with

copper-free 0.1 × NMS were referred to as 'copper-free'. About seven days growth was required for colonies to reach approximately 2 mm in diameter.

Chemostat cultures of methanotrophs were grown at 30 °C in a 2 litre fermenter (L. H. Engineering) with an agitation speed of 500 rpm, and gas flow rates of 60-80 ml min^{-1} for methane and 80-100 ml min^{-1} for air. pH was maintained at 6.8 *via* the automatic addition of 0.5 M HCl or 0.5 M NaOH. A 10 % (v/v) inoculum of culture that had grown to an OD_{540} of 0.6 in 50 ml batch cultures was used.

2.4.4 Purification and maintenance of methanotrophs

Methanotrophs were routinely checked for purity by streaking on to nutrient agar (NA) plates (Sambrook *et al.*, 1989) and incubating aerobically at 30 °C for three days. Since methanotrophs have an obligate requirement for methane, growth on NA indicated contamination. Phase contrast microscopy (Section 2.16.2) was used to examine plate cultures.

2.4.5 Antibiotics

Stock concentrations of antibiotics were prepared as detailed in Table 2.3 and aliquoted into 1 ml volumes and stored at - 20 °C. Antibiotics were filter sterilised as described in Section 2.3.

Table 2.3: Preparation of antibiotics.

Antibiotic	Dissolved in	Stock concentration ($mg\ ml^{-1}$)	Working concentration ($\mu g\ ml^{-1}$)
Ampicillin	Water	100	100
Kanamycin	Water	50	50
Streptomycin	Water	50	25
Tetracycline	Ethanol	10	10 - 12.5
Chloramphenicol	Ethanol	25	50 - 500

2.5 General purpose buffers/solutions

Agarose gel-loading buffer (6×)

Bromophenol blue	0.0125 g
Ficoll (Type 400)	0.75 g
Distilled water	5 ml

TE buffer (pH 8.0)

Tris-HCl	10 mM
di-sodium EDTA	1 mM

Tris-borate EDTA (TBE) buffer (pH 8.0)

Tris-base	90 mM
Boric acid	90 mM
EDTA	2.5 mM

Standard saline citrate (SSC) (20 ×)	
NaCl	34.66 g
tri-sodium citrate	17.64 g

These were dissolved in 150 ml of distilled water and the pH was adjusted to pH 7.0 with 10 M NaOH and the volume was made up to 200 ml.

2.6 Conjugation and transformation

2.6.1 *E. coli* transformation by chemical competence

Chemically competent cells were used for transformation of ligation reactions and plasmid constructs greater than 10 kb in size. A 1 % inoculum from an overnight culture of *E. coli* was (aseptically) added to 200 ml SOB medium (2 % (w/v) bacto-tryptone; 0.5 % (w/v) yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$) pre-incubated at 37 °C. After incubation at 37 °C, at an OD_{540} of 0.4, cells were chilled on ice for 10 min, harvested and resuspended in solution RF1 (100 mM RbCl, 50 mM $MnCl_2.4H_2O$, 30 mM potassium acetate, 10 mM $CaCl_2.2H_2O$, 15 % (v/v) glycerol, pH 5.8). Cells were harvested and resuspended in solution RF2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2.2H_2O$, 15 % (v/v) glycerol, pH 6.8) and incubated on ice. 100 µl cell aliquots were frozen in liquid nitrogen and stored at -70 °C until required.

100 µl aliquots of competent cells were thawed on ice and mixed with 0.5 µg of DNA in a volume of 10 % (v/v), gently mixed and incubated on ice for 30 min. Heat shock at 42 °C for 90 s was followed by the addition of 800 µl SOC (2 % (w/v) bacto-tryptone; 0.5 % (w/v) yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) and incubation at 37 °C with agitation for 1 h. This

was to allow time for plasmid-encoded antibiotic resistance genes to be expressed. Cells were then plated on to LB agar containing an appropriate antibiotic for plasmid selection.

2.6.2 Electrotransformation

(i) *E. coli*

A Bio-Rad Gene Pulser was used for electroporation according to the manufacturer's instructions. A 1 ml aliquot from an overnight culture of *E. coli* was washed three times in sterile distilled water and resuspended in 40 µl of sterile distilled water. Approximately 100 ng of plasmid DNA was added to the cell suspension followed by electrotransformation using 0.2 cm electroporation cuvettes at a field strength of 12.5 kV cm^{-1} and a time constant of 5 ms. 1 ml SOC was rapidly added to the transformed cells followed by incubation at 37 °C with agitation for 1 h.

(ii) *P. putida*

A Bio-Rad Gene Pulser was used for electroporation according to the method of Smith and Iglewski (1989). A 50 ml culture of *P. putida* was grown in LB at 30 °C to an OD_{540} of 0.3 - 0.5. Cells were harvested at 7,000 × *g* for 10 min at 4 °C. The pellet was washed twice in 50 ml of 300 mM sucrose and resuspended in 500 µl of 300 mM sucrose. Cells were chilled on ice for 30 min and 40 µl of the cell suspension mixed with an appropriate amount of plasmid DNA. Electrotransformation was performed using 0.2 cm electroporation cuvettes at a field strength of 8.0 - 12.5 kV cm^{-1} and a time constant of 5 ms. 3 ml LB was added immediately to the transformed cells, followed by incubation for 2 h at 30 °C.

2.6.3 Methanotroph conjugations

The technique used for conjugating plasmids from *E. coli* into methanotrophs was based upon a method developed by Martin (1994). Overnight cultures of methanotrophs were used, which were at an OD_{540} of about 0.2. Appropriate volumes of methanotroph recipient and overnight donor *E. coli* culture containing the plasmid to be conjugated were washed with NMS. They were collected on a 47 mm sterile nitro-cellulose filter (0.2 μm pore size; Millipore). The filter was placed on NMS agar that contained 0.02 % (w/v) Proteose Peptone (Difco) and incubated for 24 h at 30 °C in the presence of methane. Cells from the conjugation plates were resuspended in 10 ml of NMS by vortexing. The filter was removed and cells concentrated by centrifugation in a Mistral 2000 swing rotor (MSE, Loughborough, UK) at 7,000 × *g* for 5 min.

The cell pellet was resuspended in 1 ml NMS and 100 μl aliquots plated onto selective agar, followed by incubation for two to three weeks with methane/air (1:1 (v/v)) until single colonies appeared.

2.7 Nucleic acid techniques

2.7.1 Small scale plasmid extraction by alkaline lysis

(i) *E. coli* and *P. putida*

This procedure was performed according to the method of Saunders and Burke (1990).

(ii) Methanotrophs

This procedure was based on the method of Saunders and Burke (1990). 50 ml of a methanotroph culture (OD_{540} = 0.5-0.8) was pelleted and resuspended in 1.8 ml Solution 1 (50 mM glucose, 20 mM Tris-HCl, 10 mM EDTA, pH 8.0) plus 200 µl of lysozyme (10 mg ml^{-1}). The suspension was incubated at 37 °C for 15 min. 4 ml of Solution 2 (0.2 M NaOH, 1 % (w/v) SDS) was added and the suspension incubated for 15 min on ice. 4 ml of Solution 3 (3 M potassium acetate, pH 4.8) was then added and the mixture inverted every 2 min for 10 min (on ice). The cell lysate was centrifuged at 21,000 × *g* for 15 min and the supernatant filtered through 4 layers of cheese cloth. 0.6 volumes of isopropanol were added and the suspension centrifuged at 39,000 × *g* for 15 min at room temperature. The pellet was rinsed in 70 % ethanol and after drying, was resuspended in a minimal volume of sterile water.

2.7.2 Large scale plasmid extraction by alkaline lysis

This procedure was based on the method of Saunders and Burke (1990), except that it was scaled up to 1 litre and a CsCl ethidium bromide gradient was used to purify the plasmid DNA further. The nucleic acid pellet (prepared as described in Section 2.7.1) was resuspended in a minimum volume of TE buffer pH 8.0, and 1 g ml^{-1} CsCl added. 0.8 ml of ethidium bromide (10 mg ml^{-1}) was added per 10 ml of DNA/CsCl solution and the mixture sealed in a quick-seal tube (Beckmann, Buckinghamshire, UK). The mixture was centrifuged at 196,000 × *g* for 16 h at room temperature in a Beckmann Vti50 rotor. This generated a gradient in which different types of DNA were separated according to differences in their buoyant densities. Bands were visualised with a UV trans-illuminator and the lower band (containing

covalently closed circular plasmid DNA) removed from the gradient with a 20 ml syringe fitted with a large gauge hypodermic needle.

Ethidium bromide was removed by repeated extraction with TE-saturated butanol until the upper organic phase was colourless. DNA was then precipitated and resuspended (Section 2.7.4) in an appropriate volume of sterile distilled water.

2.7.3 Extraction of DNA with phenol and chloroform

Phenol, chloroform and isoamyl alcohol were mixed in the ratio 25:24:1 (v/v) respectively and stored at 4 °C in the dark. To extract protein from DNA containing solutions, an equal volume of phenol-chloroform-isoamyl alcohol solution was added to the DNA and mixed vigorously by vortexing. After 2 min of centrifugation at 20,800 × *g* at 4 °C, the upper aqueous phase was retained. DNA was recovered by precipitation with ethanol as described in Section 2.7.4.

2.7.4 Precipitation of nucleic acids

0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the solution containing nucleic acid and incubated for 30 min at -70 °C or for 2 h at -20 °C. Nucleic acids were collected by centrifugation at 20,800 × *g* at 4 °C for 20 min. The pellet was washed in 70 % (v/v) ethanol and air-dried before resuspension in an appropriate volume of sterile water.

2.7.5 Preparation of chromosomal DNA from methanotrophs

Chromosomal DNA from methanotrophs was prepared by CsCl-gradient centrifugation as described by Oakley and Murrell (1988).

2.7.6 Determination of concentration of nucleic acids

The concentration of nucleic acids was calculated from the absorbance at 260 nm, measured using quartz cuvettes with a 1 cm path length. A Beckmann DU-70 spectrophotometer was used, assuming an A_{260} of 1 for the following nucleic acid concentrations: oligonucleotides, 20 μg ml^{-1}; RNA, 40 μg ml^{-1} and double stranded DNA, 50 μg ml^{-1} (Sambrook *et al.*, 1989).

2.7.7 Enzymatic modification of DNA

2.7.7.1 Restriction endonuclease digestion of DNA

Restriction endonucleases and buffer solutions were supplied by Gibco BRL, Paisley, UK. DNA was digested according to the manufacturer's instructions.

2.7.7.2 Ligation of DNA using T4 DNA ligase

T4 DNA ligase was obtained from Gibco BRL and used according to the manufacturer's instructions.

2.7.7.3 Dephosphorylation of DNA

Alkaline phosphatase was obtained from Boehringer Mannheim, East Sussex, UK and was used according to the manufacturer's instructions.

2.7.7.4 Agarose gel electrophoresis

Agarose gels were made and run in 1 × TBE buffer. For routine separation of DNA a 1 % (w/v) agarose TBE gel was used. Minigel apparatus were supplied by Flowgen Instruments Ltd, Sittingbourne, UK. 0.5 μg ml^{-1} ethidium bromide was

added directly to the gel before casting. Minigels were run at a constant current of 50 mA for 1-2 h. DNA was visualised by placing the gel on a UV transilluminator. Gels were photographed using an instant camera (CU5 Land Camera, loaded with Polaroid 665 positive /negative film). 1 kb ladder (Gibco BRL) was used to calibrate agarose gels.

2.7.7.5 Elution of DNA from agarose

DNA fragments separated by agarose gel electrophoresis were removed from the gel by excision of agarose and electroeluted. Dialysis tubing was prepared by boiling in sodium bicarbonate (2 % w/v) and 1 mM EDTA, pH 8.0 and then sterilised by autoclaving. The dialysis tubing was stored at 4 °C and rinsed with distilled water prior to use. The excised agarose was placed in prepared dialysis tubing containing 0.5 × TBE and electrophoresed at 45 mA for 30 min. The current was then reversed for 30 s to free DNA retained on the tubing wall. Ethidium bromide was removed by extraction with TE-saturated butanol, and the lower aqueous phase retained. The DNA solution was phenol:chloroform extracted (Section 2.7.3), precipitated with ethanol and resuspended in a minimal volume of water.

2.8 Nucleic acid hybridisation

2.8.1 Southern blot hybridisation

Southern blot hybridisations were performed as described in Sambrook *et al.* (1989). The conditions used for hybridisation and washing of nylon membranes (Amersham, Little Chalfont, UK) containing DNA after Southern blotting were essentially as described by Oakley and Murrell (1988).

2.8.2 Colony blot hybridisation

Transformed clones were transferred onto a nylon Hybond-N membrane (Amersham) by overlaying it onto an LB-agar plate (23 cm × 25 cm), containing the appropriate antibiotic(s). The membrane was incubated at 37 °C overnight to allow growth of the clones on the membrane. 2 sheets of 3M paper (Whatman, Maidstone, UK) were placed into each of four trays containing: (1) 10 % SDS; (2) denaturing solution (0.5 M NaOH, 1.5 M NaCl); (3) neutralising solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl); (4) 6 × SSC. The membrane was placed in turn into each tray for 15 min. The membrane was then air dried for 30 min before fixing the DNA with a Ultraviolet (UV) Stratalinker (Stratagene, Cambridge, UK). The conditions used for hybridisation and washing of membranes carrying DNA after colony blotting were as described by Oakley and Murrell (1988).

2.8.3 Radioactive labelling of DNA by nick translation

Nick translation of double-stranded DNA was performed using ^{32}P dCTP and/or ^{32}P dGTP. Approximately 250 ng of DNA was mixed with 4 µl of 10 × nick translation buffer (0.5 M Tris-HCl pH 7.5, 0.1 M $MgSO_4$, 1 mM DTT, 500 µg ml^{-1} bovine serum albumin), 20 nmol of deoxynucleotide mix (containing dATP, dCTP, dGTP and dTTP needed for polymerisation), 1 µl of DNAase I (8 fg ml^{-1}) (Sigma Chemical Co, Poole, UK), 10 µCi ^{32}P dCTP and /or ^{32}P dGTP (Amersham) and 2.5 units of DNA polymerase I (Gibco BRL).

Nick translation reactions were carried out at 14 °C for 4 h and labelled DNA removed from unincorporated nucleotides using a Sephadex G-25 column (Pharmacia Biotech) (Section 2.8.4).

2.8.4 Removal of unincorporated label

A Sephadex G-25 column (Pharmacia Biotech) poured inside a 5 ml plastic pipette was equilibrated with TE buffer. The nick translation mix was applied to the column and 0.8 ml fractions were collected. The radioactivity of the fractions was determined using a Geiger Müller counter, which showed two peaks of activity. The first peak was due to labelled DNA and the second peak was due to unincorporated nucleotides. The purified probe DNA was denatured by heating at 100 °C for 5 min, followed by cooling on ice immediately prior to use.

2.9 PCR

2.9.1 Design of oligonucleotides

Oligonucleotides for use in PCR, double stranded sequencing and hybridisations were synthesised on a DNA synthesiser (Applied Biosystems, California, USA) by L. Ward (University of Warwick, UK) and purified by high performance liquid chromatography (HPLC).

2.9.2 PCR amplification

PCR amplification was performed in a total volume of 50 µl in 0.5 ml Eppendorf tubes under a layer of 50 µl of mineral oil using a 480 DNA thermal cycler (Perkin Elmer, Norwalk, USA). Each reaction contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 10 µM of each dNTP, 50 pmol of each primer, 2.5 Units of *Taq* polymerase (Gibco BRL) and approximately 1 ng template DNA. Reaction conditions were as follows: 94 °C for 5 min; *Taq* polymerase added; 30 cycles consisting of denaturation at 94 °C for 1 min, annealing for 1 min (temperature

varied with each set of primers used) and polymerisation at 72 °C for 1 min (longer time allowed for amplification of fragments 2 kb or larger); and a final step of 72 °C for 5 min.

2.10 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Unique Site Elimination (U.S.E.) kit (Pharmacia Biotech) according to the manufacturer's instructions. Annealing of HPLC-purified primers to plasmid DNA and synthesis of a mutant DNA strand was achieved by incubating 0.025 pmol of plasmid DNA, 1.25 pmol of selection primer, 1.25 pmol of target mutagenic primer and 10 × One-Phor-All buffer plus at 100 °C for 5 min, to denature the plasmid DNA. 7 µl nucleotide mix (2.86 mM each dATP, dCTP, dGTP; 4.34 mM dATP) and 3 µl reaction mix (1,000 Units ml^{-1} T4-DNA ligase; 1400 Units ml^{-1} DNA polymerase) were then added and reactions were incubated at 37 °C for 1 h. The reactions were stopped by incubation at 85 °C for 15 min. Wild-type plasmids which still contained the unique restriction site were linearised with that restriction enzyme. This reduced their ability to transform *E. coli*. Hence, for maximum mutagenesis efficiency, two rounds of restriction enzyme selection increased the proportion of mutant plasmids within the population. After the first round of selection, repair defective *E. coli NM522 mutS* was transformed with plasmid DNA. After the second round of selection, individual transformant colonies were grown and those that contained plasmids resistant to restriction enzyme digestion with the original enzyme were DNA sequenced to verify the mutation.

2.11 DNA Sequencing

Double stranded DNA sequencing was carried out manually by the dideoxynucleotide chain termination method using the Sequenase Version 2.0 Sequencing Kit (United States Biochemical, Cleveland, USA) and ^{35}S.

2.11.1 Polyacrylamide gel electrophoresis for double stranded DNA sequencing

Acrylamide stock	
N, N'-Methylene *bis*-acrylamide	3.35 g
Acrylamide	96.5 g
Urea	233.5 g
5x TBE	100 ml

Made up to 500 ml with distilled water. Stored at 4 °C in the dark.

Urea stock	
Urea	233.5 g
5 × TBE	100 ml

Made up to 500 ml with distilled water. Stored at 4 °C.

38 × 50 cm glass plates and gel-casting unit were supplied with the Sequi-gen sequencing system (Bio-Rad). Thermal plates and top plates were treated with Repelcote® (BDH, Poole, UK). For 8 % (w/v) gels, 68 ml acrylamide stock and 102 ml urea stock were mixed and a volume of 30 ml was removed for the casting gel.

To this, 150 µl *N, N, N', N'*-tetramethylethylenediamine (TEMED) and 150 µl 25 % ammonium persulphate (AMPS) were added immediately before pouring. To the remaining 140 ml acrylamide/urea solution, 350 µl AMPS and 140 µl TEMED were added and the gel poured immediately. Sequencing reaction mixtures were boiled for 2 min then chilled on ice before loading. 2.5 µl of each sample was loaded per well and electrophoresis was carried out at a constant power of 60 W, until the Bromophenol Blue dye band had migrated 52 cm (approximately 3-4 h). After electrophoresis, gels were fixed by soaking in 10 % (v/v) acetic acid for 15 min followed by 10 % (v/v) acetic acid/10 % (v/v) methanol for 20 min. Gels were dried using a gel drier (Bio-rad) and wrapped in plastic (Cling film) before exposure to Fuji nif RX medical X-ray film. Exposure times were typically 12-36 h. Autoradiographs were developed as described in Section 2.16.4.

Sequence information was also provided by L. Ward (University of Warwick, UK) using a Model 373A automated DNA sequencing system (Applied Biosystems), using the cycle sequencing method.

2.12 sMMO protein purification

2.12.1 Purification of sMMO

Pure hydroxylase, reductase and protein B from sMMO of *Mc. capsulatus* (Bath) were provided by R. Titmus and S. Slade (University of Warwick, UK). All fast protein liquid chromatography (FPLC) equipment, media and columns were supplied by Pharmacia Biotech Ltd. sMMO purification was performed as described by Pilkington and Dalton (1990), with the following modifications:

(i) Hydroxylase

Step 1: Q Sepharose High Performance Ion Exchange Chromatography. Material containing the hydroxylase from the diethylamino ethyl (DEAE) cellulose step was loaded onto a prepacked Q Sepharose ion exchange column equilibrated with 25 mM MOPS, pH 7.0. Protein was eluted with a 0-300 mM linear gradient of NaCl. The hydroxylase was eluted from the column at 180-230 mM NaCl as the third major peak on the FPLC trace.

Step 2: Superdex 200 Gel Filtration Chromatography. Material from Q Sepharose ion exchange chromatography containing the hydroxylase was applied to a Superdex 200 gel filtration column (60 cm × 2.6 cm) equilibrated with 25 mM MOPS, pH 7.0. Active hydroxylase was identified in the first detectable peak on the FPLC trace.

(ii) Protein B

Step 1: Mono Q Ion Exchange Chromatography. Fractions containing protein B from the DEAE cellulose chromatography step were diluted 1:1 with 25 mM MOPS, pH 7.0. The material was then applied to a Mono Q column equilibrated in the same buffer and eluted with a gradient of 150-350 mM NaCl. Active protein B was eluted at 270-330 mM NaCl.

Step 2: Superdex 75 Gel Filtration Chromatography. Active fractions were applied to a Superdex 75 gel filtration column (equilibrated with 25 mM MOPS, pH 7.0) and active protein B was eluted as the third main peak on the FPLC trace.

(iii) Reductase

Step 1: Q Sepharose High Performance Ion Exchange Chromatography. Fractions containing the reductase from DEAE cellulose chromatography were diluted 1:1 with 25 mM MOPS, pH 7.0 and applied to a Q Sepharose gel filtration column. Protein was eluted with a 300-700 mM linear gradient of NaCl and active reductase was eluted at 400-450 mM NaCl.

Step 2: Superdex 75 Gel Filtration Chromatography. Active fractions were applied to a Superdex 75 gel filtration column (equilibrated with 25 mM MOPS, pH 7.0) and active reductase was eluted as the third main peak on the FPLC trace.

Recombinant sMMO was purified from *P. putida* 11767 [pJL-sMMO] based upon the modified method of Pilkington and Dalton (1990) which is described in this Section. The method used for *P. putida* is described further in Chapter 4.

2.12.2 Affinity chromatography of GST fusion proteins

2 ml of Glutathione Sepharose 4B (Pharmacia Biotech) was transferred to a disposable column and washed with 10 ml 1 × phosphate buffered saline (PBS) (1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.0) per 1.33 ml of original slurry of Glutathione Sepharose 4B, to remove the 20 % ethanol storage solution. The soluble extract was clarified by filtration through a 0.45 μm filter (Millipore), applied to the column and washed three times with 10 bed volumes of 1 × PBS. If the recombinant protein was to be eluted as a GST-fusion protein then 1 ml Glutathione elution buffer (5 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0) per ml bed volume was used, followed by incubation at room temperature for 10 min to

elute the fusion protein. The elution and collection steps were repeated twice more and the three eluates pooled. To obtain purified recombinant protein, the GST affinity tag was removed by thrombin cleavage of the column-bound fusion protein. 2 ng of thrombin was added to the washed column per μg of fusion protein and incubated for 5 to 15 min at room temperature. Pure recombinant protein was then eluted with 25 mM MOPS, pH 7.0 whilst the GST affinity tag remained bound to the column.

2.12.3 Protein determination

Total protein concentration was determined using the protein assay reagent (Bio-Rad) with bovine serum albumin (BSA) as standard.

2.12.4 Production of anti-sera

Anti-sera were raised in New Zealand White rabbits by the subcutaneous injection of 1.5 mg aliquots of purified protein homogenised with Freund's adjuvant, by V. Cooper (University of Warwick, UK). Bleeds were taken at four weekly intervals. After storage at 4 °C for 16 h, the clear supernatant was obtained and stored in 50 μl aliquots at -20 °C.

2.12.5 Solubilisation of recombinant protein

This was performed according to the method of Hoess *et al.* (1988). Insoluble extracts were prepared as described in Section 2.13.1 and resuspended in an appropriate volume of 25 mM MOPS, pH 7.0 (buffer A) to give a protein concentration of 5 mg ml^{-1}. 0.5 ml was mixed with an equal volume of settled Q Sepharose (Pharmacia Biotech) (equilibrated in buffer A) and shaken at 4 °C for 2 h.

Samples were centrifuged at 20,800 × *g* for 5 min and the supernatant removed. The pellet containing insoluble material and Q Sepharose was eluted with 0.5 ml buffer A containing 250 to 750 mM NaCl by shaking for 15 min. Soluble material was recovered in the supernatant after centrifugation at 20,800 × *g* for 15 min.

2.13 Enzyme assays for sMMO

2.13.1 Preparation of extracts

(i) *E. coli* and *P. putida*

Cells were harvested and resuspended in a minimal volume of 25 mM MOPS, pH 7.0. Cells were lysed by two to four passages through a French pressure cell (American Instrument Company, Maryland, USA) at 20,000 lb in^{-2} (137 MPa). For volumes greater than 50 ml, a cell disrupter (Constant Systems Ltd., Warwick, UK) at a breakage pressure of 25,000 lb in^{-2} was used. Crude cell extracts were separated into soluble and insoluble fractions by centrifugation at 50,400 × *g* for 40 min at 4 °C. Samples were assayed (Section 2.13.4) immediately and stored at -70 °C.

(ii) Methanotrophs

Soluble extracts were prepared as previously described (Fox *et al.*, 1989). Particulate extracts were prepared by resuspending cells in a minimal volume of 25 mM MOPS, pH 7.0 containing 40 µM $CuSO_4$ (S. Charlton, personal communication). Cells were lysed by four passages through a French pressure cell at 20,000 lb in^{-2} (137 MPa) and centrifuged at 50,400 × *g* for 60 min at 4 °C. Samples were assayed (Section 2.13.4) immediately and stored at -70 °C.

2.13.2 Whole cell assay

The method used was adapted from Brusseau *et al.* (1990). 1 ml samples of methanotroph shake flask cultures, grown in copper-free 0.1 × NMS, were incubated in the presence of a crystal of naphthalene. Oxidation of naphthalene to a mixture of 1-naphthol and 2-naphthol after 30 min agitation at 30 °C, was detected by the addition of 100 µl of a fresh 0.2 % (w/v) solution of TOD. Naphthols were detected by the formation of a purple diazo dye which was compared to control cells from *Ms. trichosporium* OB3b grown in the presence and absence of copper and negative controls, which included sterile NMS and heat killed cell samples. The naphthalene oxidation assay was not quantitative (Martin, 1994).

2.13.3 Plate assay

This method was also adapted from Brusseau *et al.* (1990). Methanotroph cultures were grown on 0.1 × NMS copper-free agar plates. Naphthalene crystals were placed in the lid of the inverted plate which was sealed inside a plastic box and incubated at 30 °C for 30 min. A fresh 0.2 % (w/v) solution of TOD was sprayed on to the agar surface allowing visualisation of sMMO expressing colonies by purple dye formation.

2.13.4 Propylene oxidation assay

Assays were carried out according to Pilkington and Dalton (1990) in 7 ml conical flasks sealed with rubber Suba Seals (W. H. Freeman). 3 ml of the gas phase of the reaction flask was removed with a syringe and replaced with 3 ml of propylene. The flask was incubated at the appropriate assay temperature for 1 min in a gyratory

water bath and the reaction initiated by the addition of 25 µl of 100 mM NADH (ethanol free). 5 µl samples were removed from the liquid phase at 0, 1, 5 and 10 min intervals and analysed for propylene oxide by gas chromatography (GC). To assay soluble or insoluble extracts, 475 µl was used. To assay purified proteins, the hydroxylase, protein B and the reductase were each added to the reaction flask. To assay one of the sMMO components, saturating amounts of the other two sMMO components was added to the reaction flask.

Commercially available NADH contains high levels of ethanol that interfere with the gas chromatographic analysis of propylene oxide. Ethanol was therefore removed from NADH by R. Titmus and S. Slade (University of Warwick, UK) using ether extraction of the NADH solution followed by evaporation under vacuum to remove residual ether.

GC was performed using a 1 m × 4 mm glass column containing Porapak Q (Phase Separations Ltd., Deeside, UK) and maintained at 190 °C with nitrogen as carrier gas at a flow rate of 30 ml min^{-1}. A flame ionisation detector was used to detect the products of the assay and the peak area was determined by a 3390A Integrator (Hewlett Packard, Berkshire, UK). The gas chromatograph was calibrated by injection of 5 µl of a 2 mM solution of propylene oxide. MMO activities were expressed as nmol of propylene oxide produced min^{-1} mg $protein^{-1}$.

2.14 Polyacrylamide gel electrophoresis (PAGE)

Soluble and insoluble extracts of *E. coli*, *P. putida* and methanotrophs were prepared as described in Section 2.13.1. For SDS-PAGE, samples were diluted with an equal volume of SDS sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT,

4 % (w/v) SDS, 0.2 % (w/v) Bromophenol Blue, 20 % (v/v) glycerol) and boiled for 5 min before loading. For native-PAGE, SDS and DTT were omitted from the sample buffer and samples were not boiled prior to loading.

For the rapid analysis of proteins from *E. coli* by SDS-PAGE, a whole cell extraction method was used. Cultures were diluted in 25 mM MOPS, pH 7.5 to an OD_{540} of 1.0. 1 ml of culture was then harvested and resuspended in 100 µl of SDS-sample buffer. After boiling for 5 min, 10 µl aliquots were analysed by SDS-PAGE. This method was not suitable for *P. putida* or methanotrophs because insufficient separation was achieved due to smearing of the proteins.

2.14.1 Preparation of SDS- and native-PAGE gels

A discontinuous system as described by Laemelli (1970) was used that comprised a 4 % (w/v) stacking gel and 12 % (w/v) separating gel.

Stacking gel

1 ml 50 % (w/v) acrylamide/*N, N'*-Methylene *bis*-acrylamide (29:1 v/v)

4.2 ml stacking gel buffer (0.375 M Tris-HCl, pH 6.8)

125 µl 10 % (w/v) SDS (omitted for native-PAGE)

6.3 ml H_2O

5 µl TEMED

1 ml catalyst (5 % (w/v) AMPS)

Separating gel

6 ml 50 % (w/v) acrylamide/*N, N'*-Methylene-bis-acrylamide (29:1 w/v)

9.4 ml separating gel buffer (1 M Tris-HCl, pH 8.8)

250 µl 10 % SDS (w/v) (omitted for native-PAGE)

4 ml 50 % (w/v) sucrose

4.8 ml H_2O

6.25 µl TEMED

625 µl catalyst (5 % (w/v) AMPS)

Running buffer comprised 2.9 g Tris base, 14.4 g glycine, 1 g SDS (omitted for native-PAGE) in 1 litre of distilled water. Electrophoresis was performed in a X-Cell II™ Mini-Cell (Novex, San Diego, USA) at 125 V constant for 1-2 h. Proteins were visualised using Coomassie Brilliant Blue (0.5 g Coomassie Blue R-250, 225 ml methanol, 45 ml glacial acetic acid, 225 ml water), and de-stained in 40 % (v/v) methanol plus 10 % (v/v) glacial acetic acid.

SDS-PAGE gels were calibrated using Mark 12 molecular mass markers (Novex), aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa. Native-PAGE gels were calibrated using high molecular mass standards (Pharmacia), albumin, 67 kDa; lactate dehydrogenase, 140 kDa; catalase, 232 kDa; ferretin, 440 kDa; thyroglobulin, 669 kDa.

2.14.2 Western blotting

Following electrophoresis, the polyacrylamide gel was soaked for 10 min in transfer buffer (20 mM Tris-HCl, pH 8.0, 150 mM glycine, 20 % (v/v) methanol). Proteins were electroblotted on to Hybond-C nitrocellulose (Amersham) in a X-Cell II blot module (Novex) at 30 V constant for 1-2 h. The nitrocellulose was washed in distilled water and proteins were visualised by staining with 0.5 % (w/v) Ponceau Red in 5 % (w/v) trichloroacetic acid for 5 min. The position of the molecular mass markers were recorded by marking them with a pencil and the Ponceau Red removed by washing in TBS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). The filter was blocked with 20 ml TBS containing 2 % (w/v) Marvel milk powder for 2 h on a shaking platform. This was removed and a fresh 20 ml of TBS plus 2 % (w/v) Marvel milk powder was added containing 50-100 µl of the primary anti-serum.

After shaking overnight, the primary anti-serum was removed by washing three times in 20 ml TBS containing 0.1 % (v/v) Tween 20. 50 µl anti-rabbit IgG peroxidase conjugate (Sigma Immuno Chemicals) was used as the secondary antibody in 20 ml TBS plus 0.1 % (v/v) Tween 20 and shaken for 2 h. The filter was then washed in TBS plus 0.1 % (v/v) Tween 20 and twice in TBS. To visualise the bound antibody-peroxidase conjugate, the filter was stained for 10 min in staining solution and then dried at room temperature prior to storage.

The staining solution comprised the following: 1.5 g NaCl and 1 ml of 1 M Tris-HCl, pH 7.5 were dissolved in 50 ml distilled water (solution A). Solution B contained 30 mg chloronaphthol dissolved in 10 ml methanol and diluted to 50 ml in distilled water. 50 µl of 8 M hydrogen peroxide was added to solution B which was then combined with solution A immediately prior to use. To stop further staining of

the Western blot, the nitrocellulose was rinsed three times in 100 ml distilled water and left to dry for 30 min at room temperature.

2.15 Electrospray ionisation mass spectrometry (ESI-MS)

ESI-MS was performed by Alan Millar and Veronique Legros (University of Warwick, UK). A Fisons Quattro II triple quadrupole mass spectrometer (VG Biotech, Cheshire, UK) at the University of Warwick and a Quattro II triple quadrupole mass spectrometer (Micromass, Cheshire, UK) at the University of Manchester were used. Calibration was achieved using 20 pmol μl^{-1} of horse heart myoglobin (Sigma) for accurate determination of molecular masses. The electrospray carrier solvent was water/acetonitrile (1:1 v/v) containing 1 % formic acid and was applied at a flow rate of 5 μl min^{-1}. Mass spectra were acquired over the range *m/z* 600-1700 during a 10 s scan and data were collected in multichannel analyzer mode. Data processing was performed with MassLynx (VG Biotech) software. Maximum entropy processing of conventional electrospray mass spectra of protein samples was performed using the MaxEnt program incorporated in the VG MassLynx software. Theoretical molecular masses were calculated using the ExPASy world wide web molecular biology server of the Geneva University Hospital and the University of Geneva (http://expasy.hcuge.ch/).

2.16 General techniques

2.16.1 Spectrophotometry

Culture densities were measured using a Pye-Unicam SP1800 spectrophotometer at 540 nm. For optical densities greater than 0.9, dilutions to an

OD_{540} of less than 0.7 were made and the original OD_{540} was calculated from the dilution ratio.

2.16.2 Microscopy

Routine light microscopy was carried out using a Kyoga-Unilux III (Tokyo) phase contrast microscope. Microbial cultures were examined using a 1000 × magnification under oil immersion. Light photomicrographs were produced using a Zeiss Axioskop (Zeiss, Germany) phase contrast microscope (1000 × magnification).

2.16.3 Photography

All photographs were taken with Kodak T-max 100 black and white film using a Minolta X-300 35 mm camera. Negatives were developed and fixed in accordance with the manufacturer's instructions, using Kodak developer and Uni-fix fixer solutions respectively. Negatives were printed onto Kodak F3 paper and developed and fixed according to the manufacturer's instructions.

2.16.4 Autoradiography

Fuji nif RX medical X-ray film was used for all autoradiographs. Radioactive membranes or gels were exposed to this film in light-tight autoradiography cassettes with two intensifying screens. Cassettes were stored at -70°C, the length of exposure being dependent upon the signal intensity. Autoradiographs were developed and fixed according to the manufacturer's instructions.

CHAPTER 3

Heterologous expression of sMMO from *Methylococcus capsulatus* (Bath) in *Escherichia coli*

3.1 Introduction

The simplest organism for the development of an expression system is *E. coli* because it has been extensively characterised and a large number of strains and plasmids are now available. Many different types of *E. coli* expression vectors have been constructed which differ mainly in the type of promoter that they contain (Weickert, 1996). The ideal promoter for the expression of recombinant proteins in *E. coli*: (1) directs efficient transcription to allow high-level protein production; (2) is tightly regulated to reduce the metabolic burden and toxicity to the cell; (3) is inducible by a low cost chemical or by shifting growth conditions. However, no such 'ideal' promoter exists and they all have problems such as a deleterious effect on the growth of the cell (Weickert, 1996). However, a popular choice of expression system utilises bacteriophage T7-RNA polymerase (Studier and Moffatt, 1986). T7-RNA polymerase is highly selective for its own promoters, which do not occur naturally in *E. coli*. Consequently, only a small amount of T7-RNA polymerase expressed from a cloned copy of T7 gene *1* is required for high level transcription from a T7 promoter on a multicopy plasmid. Expression levels as high as 50 % of the total cell protein have been reported (Studier and Moffatt, 1986). In addition, many T7 based expression vectors are available and low temperature induction is possible. However, the problems with this type of expression system include leaky expression and the difficulty in achieving high cell densities and hence high levels of protein production (Weickert, 1996).

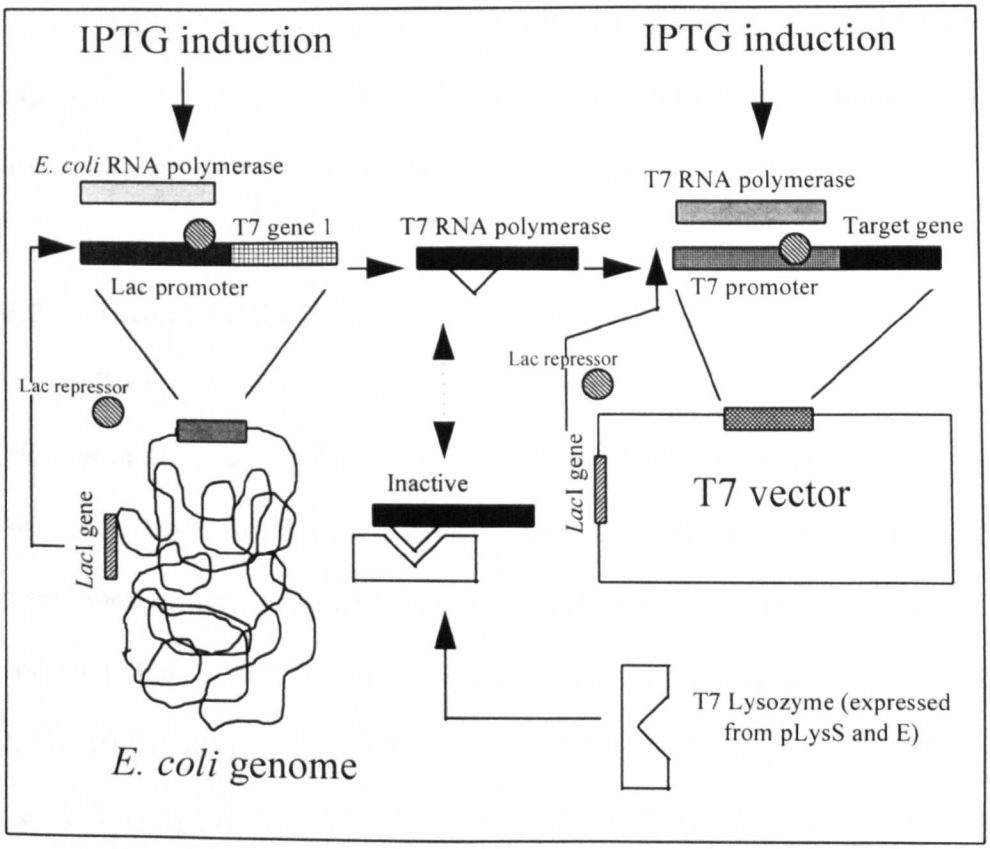
Although the principle of achieving heterologous expression of active proteins in *E. coli* is straightforward, the reality is often very different. Many proteins are complex with prosthetic groups and post-translational modifications being necessary

for functional expression. In addition, many proteins expressed in *E. coli* accumulate in the cell as insoluble, inactive inclusion bodies (Kane and Hartley, 1988; Hockney, 1994). However, *E. coli* is still considered as a good starting point for the heterologous expression of proteins.

Following the cloning and sequencing of the genes encoding sMMO from *Mc. capsulatus* (Bath) (Stainthorpe *et al.*, 1989, 1990) over-expression of these sMMO genes in *E. coli* was attempted. The genes encoding protein B and the reductase of sMMO from *Mc. capsulatus* (Bath) were expressed in *E. coli* using the T7 expression system (West *et al.*, 1992). Protein B and the reductase were functionally expressed in *E. coli* and enzyme activities were comparable to the purified proteins from *Mc. capsulatus* (Bath). However, attempts to over-express the hydroxylase were not successful, reasons for which were unknown but could have been due to the inability of *E. coli* to assemble this more complex protein (West *et al.*, 1992).

Previous attempts to over-express the sMMO genes in *E. coli* using the T7 expression system had been based upon the co-expression of pGP1-2 (West *et al.*, 1992). This system utilises heat shock to induce expression of T7-RNA polymerase (Tabor, 1990). *E. coli* BL21(DE3) (Novagen) has provided an alternative method to over-express genes using the T7-expression system (Figure 3.1). *E. coli* BL21(DE3) contains a chromosomal copy of T7-RNA polymerase gene *1* under the control of an isopropyl β-D-thiogalactopyranoside (IPTG) inducible promoter (Tabor and Richardson, 1985). Therefore, growth temperatures and IPTG concentrations can be easily optimised to maximise the amount of recombinant soluble protein in the cell (Schein, 1989). In addition, *E. coli* BL21(DE3) is deficient in both *lon* and *ompT* proteases. *E. coli* BL21(DE3) pLysS contains a plasmid that encodes T7 lysozyme,

Figure 3.1: Control elements of T7 expression in *E. coli* BL21(DE3). Figure adapted from Novagen 1996-97 catalogue.



which is a natural inhibitor of T7-RNA polymerase. This reduces transcription of target genes in non-induced cells, affording tighter control of basal expression levels. *E. coli* BL21(DE3) pLysE also encodes T7 lysozyme but expresses larger amounts and so offers even tighter control over basal expression levels which is important when over-expressing toxic genes in *E. coli*.

The genes encoding sMMO from *Ms. trichosporium* OB3b have also been cloned and sequenced (Cardy *et al.*, 1991). Expression of these genes in *E. coli* BL21(DE3) resulted in inactive sMMO (Jahng *et al.*, 1996). Functional expression of

these sMMO genes has also been reported in *P. putida* F1 (Jahng and Wood, 1994; Jahng *et al.*, 1996); attempts to repeat this work are presented in Chapter 4.

The aim of the work presented in this Chapter was to develop a heterologous expression system for sMMO in *E. coli* so that site-directed mutagenesis could be used to investigate the mechanism of methane oxidation.

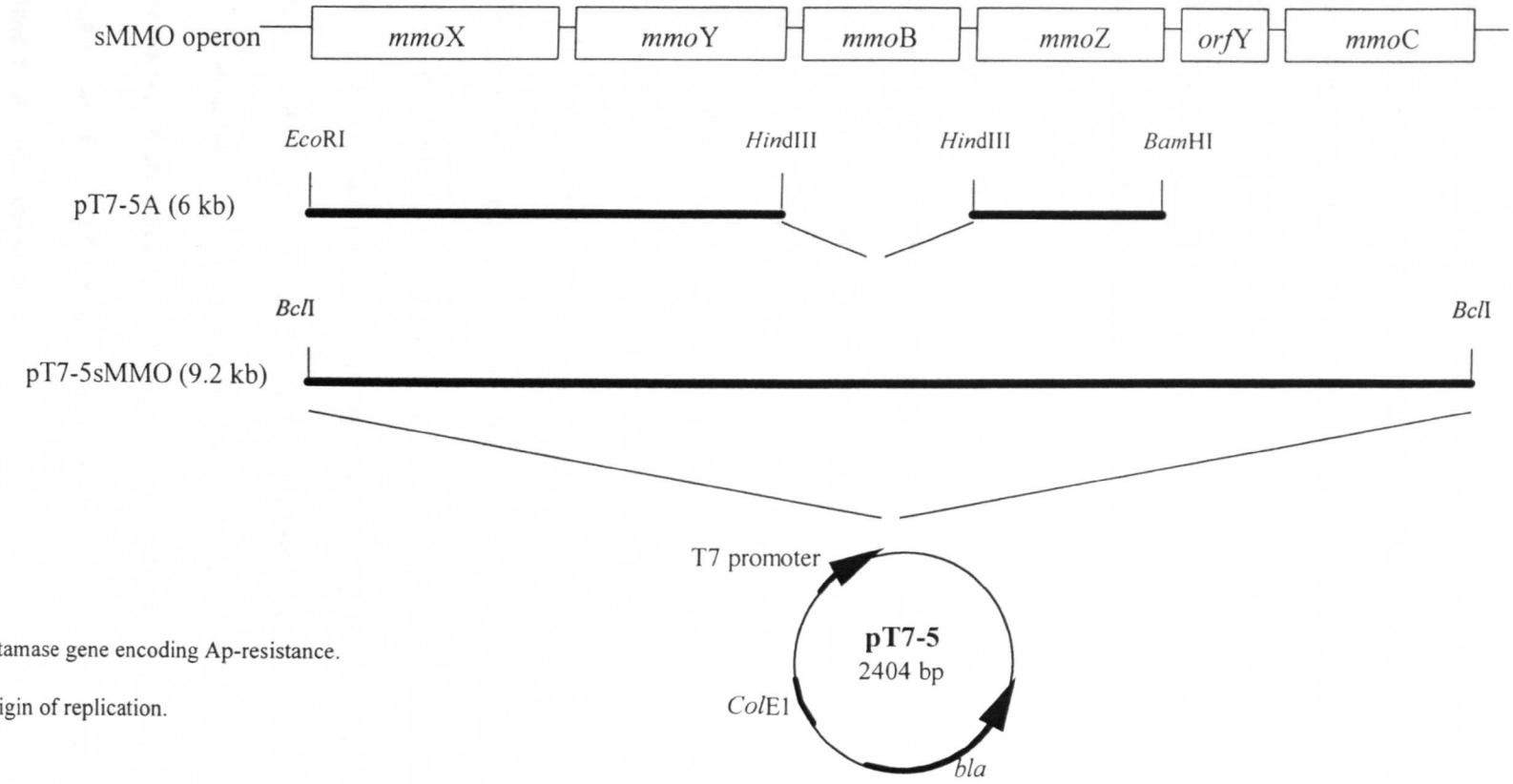
3.2 Transformation of *E. coli* for expression studies

Plasmids were constructed previously for the expression of sMMO genes from *Mc. capsulatus* (Bath) in *E. coli*, using the T7-RNA polymerase expression system (C. West, University of Warwick, UK) (Figure 3.2). Integrity of the plasmids was determined by restriction endonuclease digestion with *Bam*HI, *Eco*RI, *Hinc*II, *Pst*I and *Xho*I. For the purposes of the work described in this Chapter, *E. coli* BL21(DE3), *E. coli* BL21(DE3) pLysS and *E. coli* BL21(DE3) pLysE, were transformed with pT7-5sMMO and pT7-5A by electroporation. The sMMO-negative control used in all cases was *E. coli* BL21(DE3) [pT7-5].

3.3 Preparation of anti-serum against the hydroxylase from *Mc. capsulatus* (Bath)

A detailed study of the expression of sMMO was undertaken in order to determine the reason for the inactivity of sMMO from *Mc. capsulatus* (Bath) when expressed in *E. coli*. Antibodies were required to monitor the levels of expression of recombinant sMMO proteins. Polyclonal antibodies to protein B and the reductase from *Mc. capsulatus* (Bath) were provided by C. West (University of Warwick, UK) but polyclonal antibodies to the hydroxylase were required. The hydroxylase from

Figure 3.2: T7 vectors for expression of sMMO from *Mc. capsulatus* (Bath) in *E. coli*. pT7-5A was by constructed by amplifying by PCR, *Eco*RI/*Hin*dIII and *Hin*dIII/*Bam*HI fragments containing *mmoX*, *mmoY* and *mmoZ*. These were then subcloned into pT7-5. pT7-5sMMO was constructed by sub-cloning a 6.8 kb *Bam*HI fragment containing the sMMO operon from pCH4 into pT7-5.



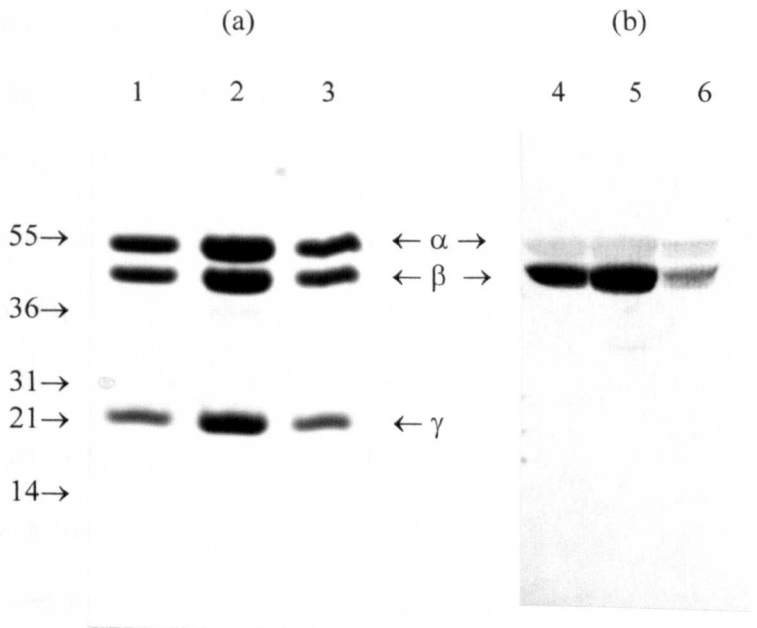
bla, β-lactamase gene encoding Ap-resistance.

*Col*E1, origin of replication.

Mc. capsulatus (Bath) was purified using the methods described in Section 2.12.1, by R. Titmus (University of Warwick, UK). To further improve the purity, material containing the hydroxylase was purified *via* two passages through a Mono Q ion exchange chromatography column. After purification, aliquots of purified hydroxylase from 40 to 80 μg were separated by SDS-PAGE and visualised with Coomassie Brilliant Blue to establish the purity (Figure 3.3a). The protein was deemed to be approximately 90 % pure and hence suitable for the raising of polyclonal antibodies in rabbits. Anti-sera were raised as described in Section 2.12.4, using native hydroxylase as the immunogen. The first bleed was taken four weeks after the first immunisation. SDS-PAGE of pure hydroxylase from *Mc. capsulatus* (Bath) followed by Western blotting with 100 μl of the first bleed of anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) was performed (Figure 3.3b).

Cross-reactivity was observed with the α and β subunits of the hydroxylase but not with the γ subunit. The reason for the lack of cross-reactivity with the γ subunit was not known but it could have been due to the use of native hydroxylase and possibly the folding of the γ subunit in such a way that immunogenic epitopes were not exposed on the protein. Subsequently, 1.5 mg of pure hydroxylase was denatured by heating at 80 °C for 5 min, which caused precipitation of the protein. This was injected into the same rabbit and another bleed was taken four weeks later. This time the serum cross-reacted with all three subunits of the hydroxylase and was subsequently used for all of the Western blots that required anti-serum against the hydroxylase from *Mc. capsulatus* (Bath). The second bleed anti-serum showed a much stronger cross-reactivity with the β and γ subunits of the hydroxylase than with the α subunit, but cross-reactivity with the α subunit was sufficient for its detection.

Figure 3.3: (a) SDS-PAGE and (b) Western blotting of purified hydroxylase from *Mc. capsulatus* (Bath). Lanes 1 to 3, 40, 80 and 60 μg respectively of pure hydroxylase from *Mc. capsulatus* (Bath); lanes 4 to 6, Western blot of lanes 1, 2 and 3 respectively, probed with the first bleed of anti-serum against the hydroxylase of *Mc. capsulatus* (Bath).

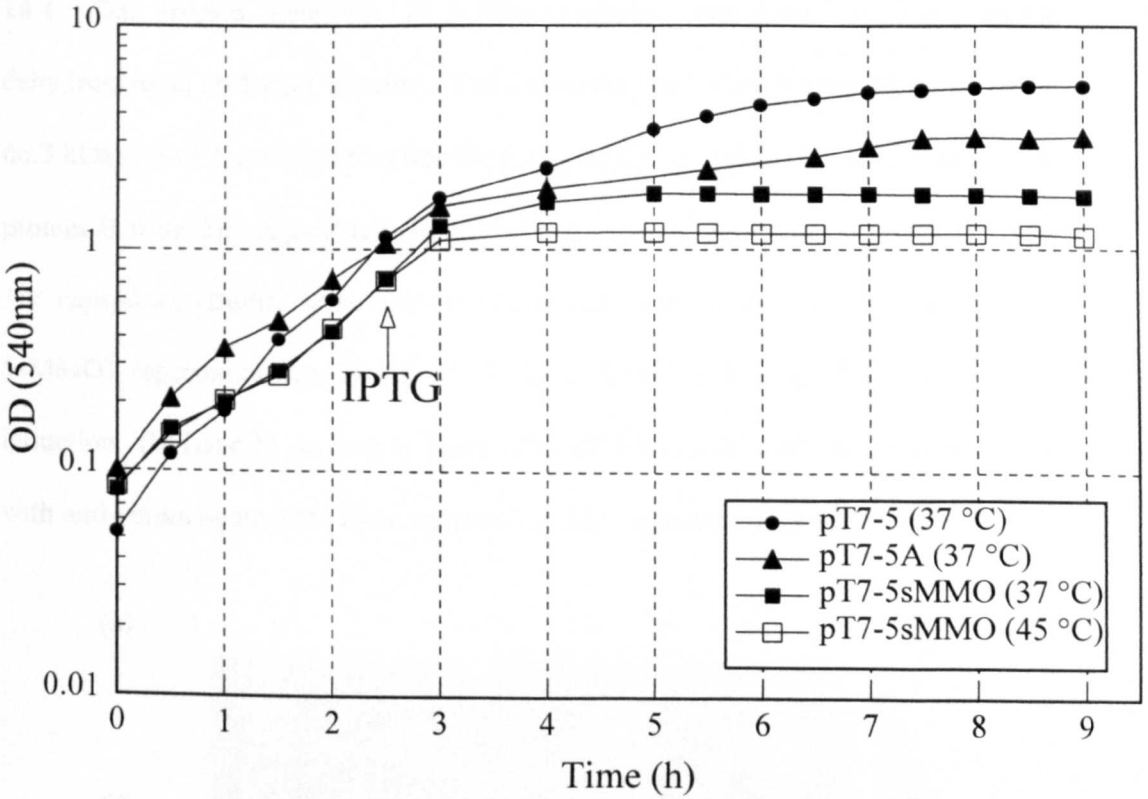


The pre-immune serum did not cross-react with pure hydroxylase protein.

3.4 Expression of sMMO in *E. coli* BL21(DE3)

sMMO expression in *E. coli* grown in LB batch culture was investigated using the plasmid pT7-5sMMO (Figure 3.2). Expression was monitored after induction of the sMMO genes with 1 mM IPTG at an OD_{540} of 0.5. As shown in Figure 3.4, the culture was induced during the exponential phase of growth, when the bacteria were in their most active stage of growth. The growth rate of *E. coli* BL21(DE3) [pT7-5sMMO] was reduced when expression of sMMO was induced. The cause of the reduced growth rate, in comparison to *E. coli* BL21(DE3) [pT7-5] was likely to be due to the high level of expression from the T7 promoter. Addition of IPTG was followed by the induction of polypeptides of sizes equal or very similar to the subunits of sMMO (Figure 3.5). Propylene oxidation enzyme assays of soluble and insoluble extracts of *E. coli* BL21(DE3) [pT7-5sMMO] were performed in the presence and absence of pure sMMO proteins to establish whether enzyme activity for recombinant sMMO proteins could be obtained (Table 3.1). The recombinant hydroxylase was inactive, but protein B and the reductase were active, confirming the results of West *et al.* (1992). Since the hydroxylase has an intrinsic requirement for iron (Fox *et al.*, 1988; Green and Dalton, 1988) the same experiment was performed using LB supplemented with 5-10 μM ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2.6H_2O$). The latter was added at the same time as IPTG to minimise natural oxidation of ferrous iron to ferric iron. Addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ to the growth medium had no effect on the growth rate and propylene oxidation assays confirmed that the recombinant hydroxylase was still inactive.

Figure 3.4: Growth of *E. coli* BL21(DE3) [pT7-5sMMO] and [pT7-5A] at 37 °C before and after IPTG induction.



Arrow indicates the addition of 1 mM IPTG at an OD_{540} of ~ 0.5. The results are the mean of three experiments. Standard error was approximately 5 %.

Whole cell lysates of *E. coli* BL21(DE3) [pT7-5sMMO] were analysed using SDS-PAGE and Western blotting at 0, 1, 2, 3, 4 and 5 h after IPTG induction. Figure 3.5 illustrates the accumulation of protein B and the reductase to about 10 % of the total cell protein after 1 h of induction. This was subsequently confirmed with Western blotting using anti-sera against protein B and the reductase (data not shown). In addition, expression of the β and γ subunits of the hydroxylase was identified, but the α subunit of the hydroxylase could not be seen using Coomassie Brilliant Blue staining. Western blotting with anti-serum against the hydroxylase confirmed

Figure 3.5: (a) SDS-PAGE and (b) Western blotting of sMMO expression in *E. coli* BL21(DE3). (a) Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, pure hydroxylase from *Mc. capsulatus* (Bath) (20 μg); lane 3, pure protein B from *Mc. capsulatus* (Bath) (15 μg); lane 4, pure reductase (15 μg) from *Mc. capsulatus* (Bath); lanes 5 to 10, whole cell lysates of *E. coli* BL21(DE3) [pT7-5sMMO] (approximately 30 μg) 0, 1, 2, 3, 4 and 5 h respectively after IPTG induction. (b) Lane 11, as lane 1; lanes 12 to 17, Western blot of lanes 5 to 10 probed with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath).

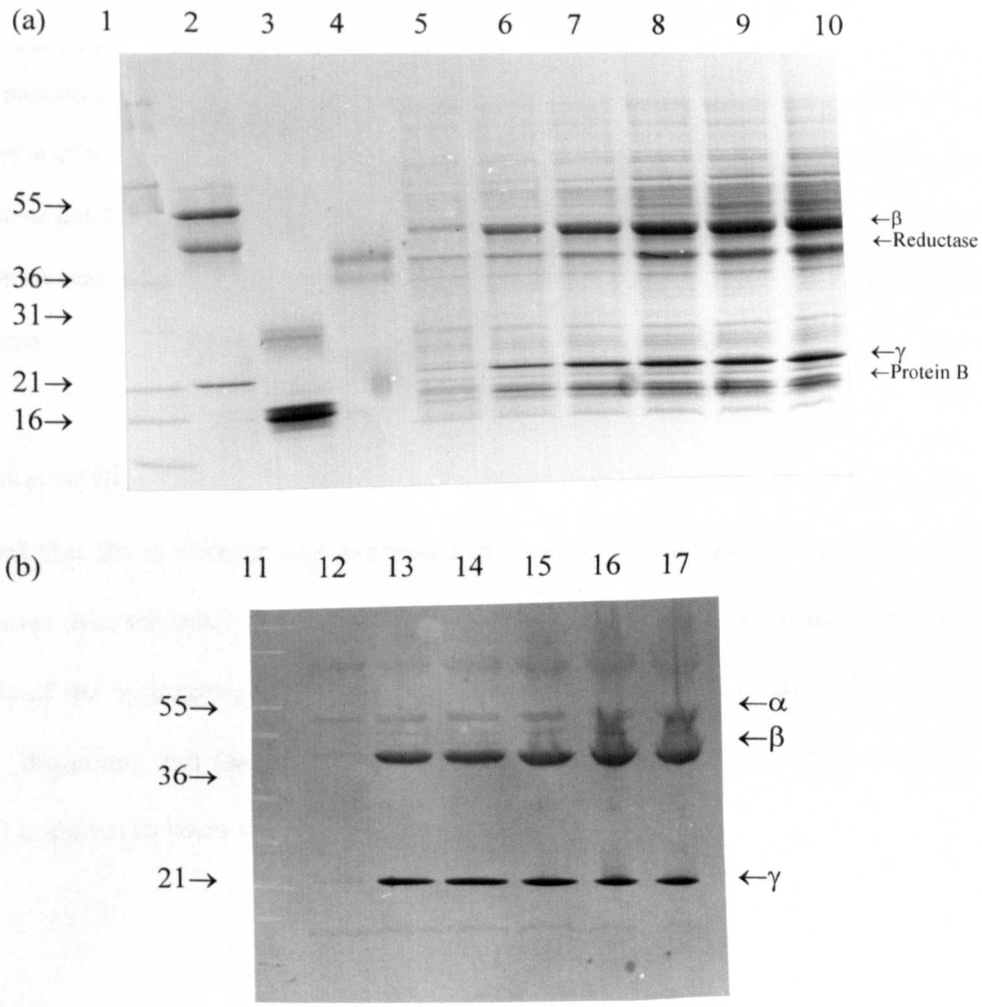


Table 3.1: Complementation of soluble extracts of *E. coli* BL21(DE3) [pT7-5sMMO] with purified sMMO proteins from *Mc. capsulatus* (Bath).

Protein	Specific activity (nmol min^{-1} mg $protein^{-1}$)
A + B + C	123 ± 2.5
E. coli BL21(DE3) [pT7-5sMMO] soluble extract	0
E. coli BL21(DE3) [pT7-5sMMO] insoluble extract	0
Soluble extract + B + C	0
Soluble extract + A + C	12 ± 0.6
Soluble extract + A + B	5 ± 0.8
Soluble extract + A	9 ± 1.5
Soluble extract + B	0
Soluble extract + C	0
B + C	0
A + C	0
A + B	0

Purified proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase. Assays were performed at 45 °C in 500 µl 25 mM MOPS, pH 7.0. 8 nmol of each of proteins A, B and C, 481 nmol of propylene and 224 µmol NADH and about 15 mg of soluble extract were used in each assay. The GC retention time of propylene oxide was 2.12 min in all assays. The results are the mean of three experiments.

expression of the β and γ subunits of the hydroxylase. Although the Western blot indicated that the α subunit was expressed in *E. coli*, its abundance was at a much lower level than the other subunits of the hydroxylase. In *Mc. capsulatus* (Bath), the subunits of the hydroxylase are expressed in equimolar amounts, as judged by SDS-PAGE (Woodland and Dalton, 1984). The negative control of *E. coli* BL21(DE3) [pT7-5] is shown in lanes 10 and 20 of Figure 3.7.

From these results, one hypothesis for the lack of enzyme activity from the recombinant sMMO may be that correct assembly could not occur within *E. coli*. Native-PAGE and Western blotting were used to determine whether the recombinant sMMO had the same molecular mass as sMMO from *Mc. capsulatus* (Bath). 1 litre cultures of *E. coli* BL21(DE3) [pT7-5] and *E. coli* BL21(DE3) [pT7-5sMMO] were grown, induced and soluble extracts were prepared as described in Section 2.13.1. Soluble extract from *Mc. capsulatus* (Bath) expressing sMMO was used as the positive control. The native-PAGE gel and Western blot (Figure 3.6) showed that the anti-serum against the hydroxylase cross-reacted with the soluble extracts of *Mc. capsulatus* (Bath) expressing sMMO. However, no cross-reactivity was seen with soluble extracts from *E. coli* BL21(DE3) [pT7-5sMMO]. This suggested that recombinant sMMO did not assemble correctly in *E. coli*. The reason for the lack of cross-reactivity may have been due to the sMMO components retaining their monomeric molecular weights. Consequently the α subunit for example which would normally form a native 250 kDa $\alpha_2\beta_2\gamma_2$ configuration, could have maintained a monomeric molecular mass of 54 kDa. The lowest molecular mass marker was 67 kDa and so it was unlikely that the sMMO components would have remained on the native-PAGE gel.

Phase contrast microscopy of *E. coli* BL21(DE3) [pT7-5sMMO] was used to estimate the accumulation of inclusion bodies within the *E. coli* culture (Table 3.2). These data suggested that the majority of the recombinant sMMO formed insoluble protein. This hypothesis was subsequently confirmed by SDS-PAGE and Western blot analysis of soluble and insoluble extracts of *E. coli* BL21(DE3) [pT7-5sMMO] (data not shown). To determine whether the assembly of recombinant sMMO was

Figure 3.6: (a) Native-PAGE and (b) Western blot analysis of sMMO expression in *E. coli* BL21(DE3). (a) Lane 1, native molecular mass standards (albumin, 67 kDa; lactate dehydrogenase, 140 kDa; catalase, 232 kDa; ferretin, 440 kDa; thyroglobulin, 669 kDa); lane 2, 50 μg of soluble extract of *Mc. capsulatus* (Bath) expressing sMMO; lane 3, 5 μg of pure hydroxylase from *Mc. capsulatus* (Bath); lane 4, 50 μg soluble extract of *E. coli* BL21(DE3); lane 5, 50 μg insoluble extract of *E. coli* BL21(DE3); lane 6, 50 μg soluble extract of *E. coli* BL21(DE3) [pT7-5A]; lane 7, 50 μg insoluble extract of *E. coli* BL21(DE3) [pT7-5A]; lane 8, 50 μg soluble extract of *E. coli* BL21(DE3) [pT7-5sMMO]; lane 9, 50 μg insoluble extract of *E. coli* BL21(DE3) [pT7-5sMMO]; lane 10, as lane 1. (b) Lanes 11 to 18, Western blot of lanes 2 to 9 probed with anti-serum against the hydroxylase of *Mc. capsulatus* (Bath).

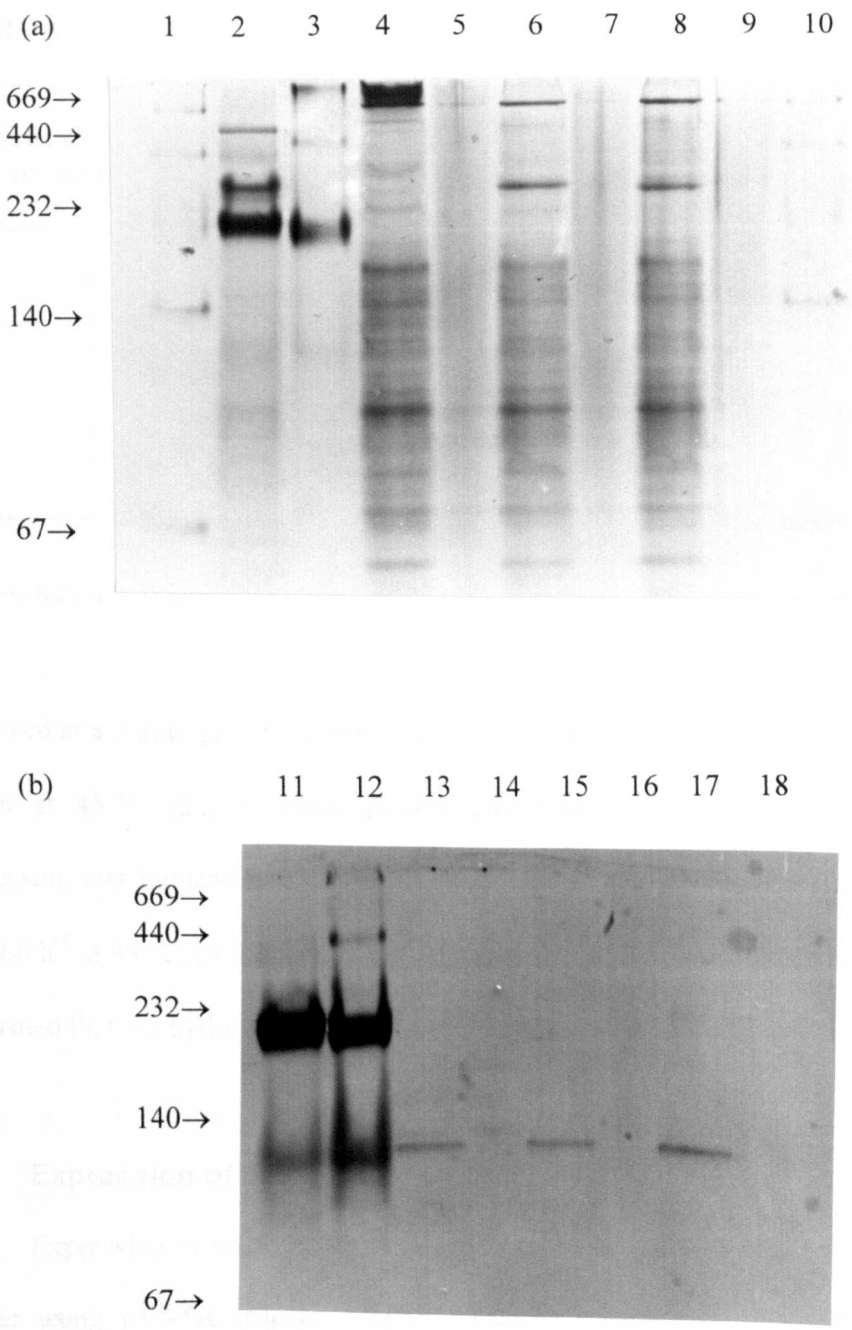

(a)
1
2
3
4
5
6
7
8
9
10
669→
440→
232→
140→
67→
(b)
11
12
13
14
15
16
17
18
669→
440→
232→
140→
67→

Table 3.2: Accumulation of inclusion bodies within *E. coli* BL21(DE3) [pT7-5sMMO].

Time after IPTG induction (h)	Inclusion bodies (%) *E. coli* [pT7-5]	Inclusion bodies (%) *E. coli* [pT7-5sMMO]
0	0	5
1	0	50
2	5	60
3	5	70
4	5	90
5	5	90

Inclusion bodies (%) refers to the percentage of cells in the culture containing inclusion bodies.

improved at a higher growth temperature, *E. coli* BL21(DE3) [pT7-5sMMO] was also grown at 45 °C (the optimum growth temperature of *Mc. capsulatus* (Bath)). Expression was induced with 1 mM IPTG at an OD_{540} of 0.5 for 5 h (Figure 3.4). μ was 0.6 h^{-1} at 45 °C, in comparison to 0.8 h^{-1} at 37 °C and propylene oxidation assays confirmed that the hydroxylase was inactive.

3.5 Expression of the hydroxylase in *E. coli* BL21(DE3)

Expression of the *mmo*X, *mmo*Y and *mmo*Z genes in *E. coli* was investigated further using pT7-5A (Figure 3.2) to establish whether the problem of inactive hydroxylase could be overcome. It has been shown that the amount of insoluble protein that accumulates within *E. coli* can be reduced by optimising both growth temperature and the amount of IPTG used to induce expression (Schein and Noteborn, 1988; Schein, 1989). This strategy was used in an attempt to maximise the amount of

soluble recombinant protein (and perhaps to obtain active recombinant hydroxylase). The results of the experiment are shown in Table 3.3. Protein expression was monitored using SDS-PAGE of whole cell lysates and soluble extracts (Figure 3.7) and enzyme activity was monitored using the propylene oxidation assay. Reduction of the growth temperature and IPTG concentration successfully reduced the accumulation of inclusion bodies within the cells. However, increased levels of the α subunit expressed in a soluble form were not seen and SDS-PAGE indicated that the decrease in inclusion body formation was concomitant with a decrease in the amount of recombinant hydroxylase expression. It therefore appeared that this strategy only succeeded in reducing inclusion body formation because less of the hydroxylase was being expressed within the cell. The amount of soluble hydroxylase was not increased using this method. After IPTG induction of *E. coli* BL21(DE3) [pT7-5A] for 3 h, cells were harvested and soluble and insoluble extracts prepared as described in Section 2.13.1. Native-PAGE and Western blot analysis of the recombinant hydroxylase (Figure 3.6) suggested that the protein did not assemble correctly in *E. coli* since no cross-reactivity was seen with proteins greater than 67 kDa in size. The native molecular mass of wild-type hydroxylase from *Mc. capsulatus* (Bath) was about 250 kDa.

Since the stability of the recombinant hydroxylase was not known, propylene oxidation assays were performed within 90 min of harvesting the cells to maximise the chance of assaying active protein. Pure protein B and pure reductase from *Mc. capsulatus* (Bath) were added to soluble extracts in an attempt to reconstitute a fully functional sMMO (Table 3.4). A reaction product with a GC retention time very similar to that of the propylene oxide control was detected from assays using soluble

Table 3.3: Effect of temperature and IPTG concentration on the expression of the α, β and γ subunits of the hydroxylase in *E. coli* BL21(DE3).

Temperature (°C)	IPTG concentration (mM)	Inclusion bodies after 3 h (%)	Protein expression[1] α	β	γ	Specific activity (nmol min^{-1} mg $protein^{-1}$)
37	1	90	+	+	+++	ND
	0.4	80	+	+	+++	10 ± 1.5
	0.1	60	+/–	+	++	0
	0.05	60	+/–	+	+	0
30	0.1	40	+/–	+	++	0
	0.05	30	+/–	+	+	0
25	0.1	20	+/–	+	++	ND
20	0.1	20	+/–	+	++	ND

[1] Protein expression is shown as a percentage of the total cell protein estimated by SDS-PAGE: +/- (< 5 %); + (5 %); ++ (10 %); +++ (15 %). Inclusion bodies after 3 h (%) refers to the number of inclusion bodies in the culture after 3 h of induction with IPTG. ND, not determined.

extracts of *E. coli* BL21(DE3) [pT7-5A], complemented with pure protein B and reductase from *Mc. capsulatus* (Bath). *E. coli* BL21(DE3) used to prepare the soluble extract for the control reaction lacked the plasmid encoding the sMMO genes and showed no detectable propylene oxidation activity. The results in Table 3.4 suggested that the recombinant hydroxylase being expressed under these conditions was active

Figure 3.7: (a) SDS-PAGE and (b) Western blotting of *Mc. capsulatus* (Bath) hydroxylase expression in *E. coli* BL21(DE3) [pT7-5A]. (a) Lane 1, 5 μg of pure hydroxylase from *Mc. capsulatus* (Bath); lanes 2 to 7, whole cell lysates of *E. coli* BL21(DE3) [pT7-5A] 0, 1, 2, 3, 4 and 5 h respectively after induction with 0.4 mM IPTG at 37 °C; lane 8, 25 μg of soluble extract of *E. coli* BL21(DE3) [pT7-5A]; lane 9, 25 μg of insoluble extract of *E. coli* BL21(DE3) [pT7-5A]; lane 10, 25 μg of soluble extract of *E. coli* BL21(DE3) [pT7-5]. (b) Lanes 11 to 20, Western blot of lanes 1 to 10 probed with anti-serum against the hydroxylase of *Mc. capsulatus* (Bath).

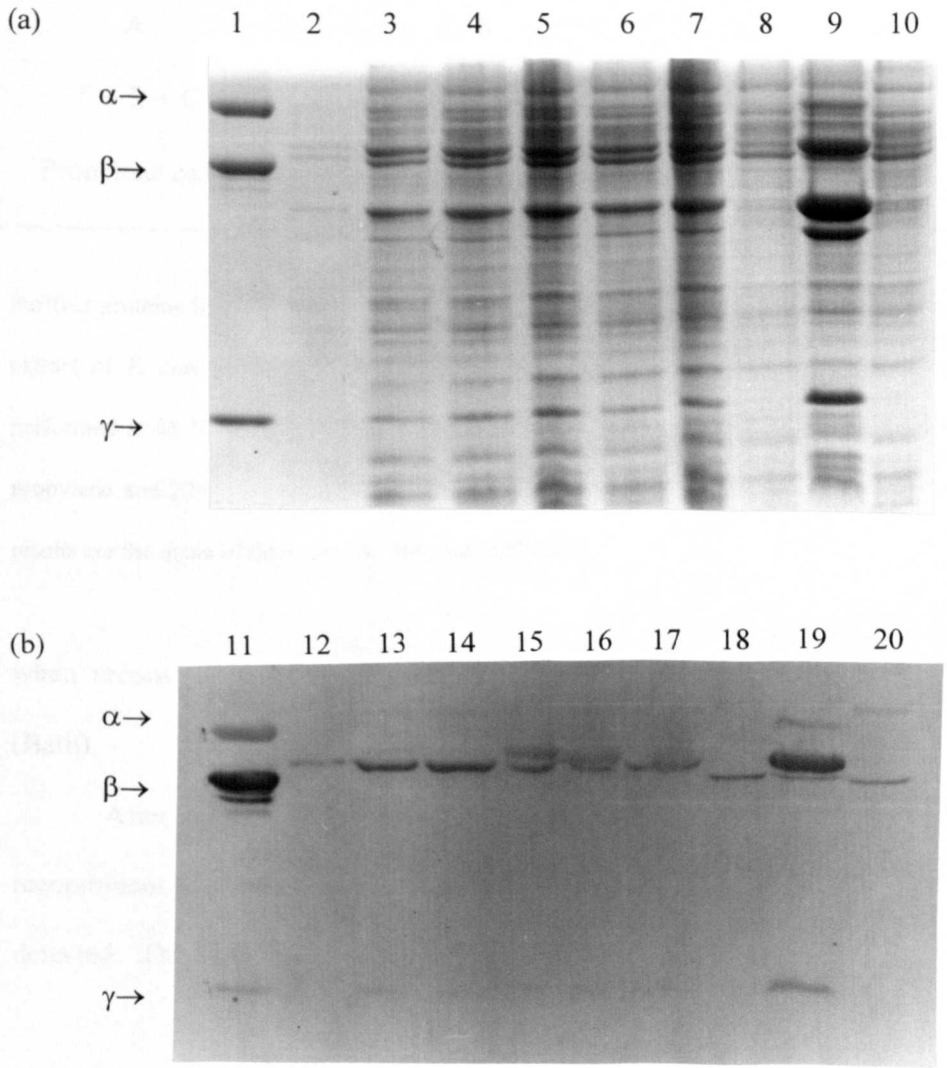


Table 3.4: Complementation of soluble extracts of *E. coli* BL21(DE3) [pT7-5A] with purified sMMO proteins from *Mc. capsulatus* (Bath).

Protein	Specific activity (nmol min^{-1} mg $protein^{-1}$)	Retention time of product (min)
A + B + C	145 ± 2.5	1.88 - 1.9
B + C	0	NA
A^1 + B + C	10 ± 1.5	1.80 - 1.83
A^1 + B	0	NA
A^1 + C	0	NA
A^1	0	NA
E + B + C	0	NA
Propylene oxide	NA	1.89

Purified proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase. A^1, soluble extract of *E. coli* BL21(DE3) [pT7-5A]; E, soluble extract of *E. coli* BL21(DE3). Assays were performed at 45 °C in 500 µl 25 mM MOPS, pH 7.0. 8 nmol of proteins A, B and C, 481 nmol of propylene and 224 µmol NADH and about 15 mg of soluble extract were used in each assay. The results are the mean of three assays. NA, not applicable.

when reconstituted with pure protein B and pure reductase from *Mc. capsulatus* (Bath).

After overnight storage at -70 °C, no propylene oxidation activity for the recombinant hydroxylase, reconstituted with pure protein B and reductase was detected. This is not suprising because during the routine purification of sMMO from

Mc. capsulatus (Bath), only whole cells are stored at -70 °C since soluble extracts lose appreciable amounts of activity after freezing and thawing (Woodland and Dalton, 1984). Therefore, the experiment was repeated with the aim of using gas chromatography-mass spectrometry (GC-MS) to confirm that the product with a retention time of 1.80-1.83 min was propylene oxide. In addition, experiments such as the use of the suicide substrate, acetylene (Prior and Dalton, 1985) would act as additional evidence for sMMO activity. However, three independent attempts to repeat this work proved unsuccessful. In all cases the LB media, *E. coli* BL21(DE3) [pT7-5A], pure sMMO proteins and growth conditions were prepared in the same way as in the original experiment but no products with a retention time similar to the propylene oxide control were seen. The reasons for the unreproducibility of this result were unclear. In an attempt to obtain more consistent recombinant hydroxylase activity, further experiments were conducted with *E. coli* BL21(DE3) [pT7-5A], as described in Sections 3.5.1 to 3.5.6.

3.5.1 Hydroxylase expression in *E. coli* BL21(DE3) pLysE and pLysS

SDS-PAGE of recombinant hydroxylase showed low levels of α subunit expression (Figures 3.5 and 3.7). One reason for this could be that the α subunit was particularly sensitive to proteolytic degradation within the cell. In *E. coli* BL21(DE3), the T7 promoter is totally inactive in the absence of T7-RNA polymerase but the latter has a high basal level of expression due to the leaky *lac* promoter that controls its expression. Using the pLysE and pLysS expression systems would afford a much tighter basal level of control and so proteolytic degradation could be reduced, by

minimising the time for which recombinant proteins were exposed to cellular proteases.

Phase contrast microscopy of induced cultures of *E. coli* BL21(DE3) pLysE [pT7-5A] and *E. coli* BL21(DE3) pLysS [pT7-5A] indicated that the numbers of inclusion bodies were similar to those observed in *E. coli* BL21(DE3) [pT7-5A]. Soluble and insoluble extracts were separated by SDS-PAGE and the highest level of hydroxylase expression was observed in *E. coli* BL21(DE3) pLysE [pT7-5A]. The amount of α subunit expression was approximately 5 % higher than that in *E. coli* BL21(DE3) pLysS and *E. coli* BL21(DE3). However, the accumulation of the α subunit was observed primarily in the insoluble extract in all three strains, confirming that the protein was being expressed as inclusion bodies. Propylene oxidation assays confirmed that the recombinant hydroxylase was inactive.

3.5.2 Optimisation of culture media for expression of soluble hydroxylase protein

All of the experiments performed previously had relied upon the growth of *E. coli* in LB. Other media were used to investigate the effect of growth media composition on hydroxylase expression and activity in *E. coli* BL21(DE3) [pT7-5sMMO] (Table 3.5). After 5 h induction with 0.4 mM IPTG at 37 °C, propylene oxidation assays were performed on soluble and insoluble extracts of cells grown in different growth media. In all cases, no sMMO activity was detected and SDS-PAGE analysis indicated that the expression levels of the recombinant hydroxylase components were very similar to those obtained in LB (data not shown).

Table 3.5: Different growth media used during expression of recombinant hydroxylase in *E. coli.*

Media	Supplement	μ (h^{-1})	Reference
LB	NA	0.82	Sambrook *et al.* (1989)
LB	4 μM $Fe(NH_4)_2(SO_4)_2.6H_2O$ [1]	0.79	This study
M9 minimal media	NA	0.32	Jahng and Wood, (1994)
M9 minimal media	4 μM $Fe(NH_4)_2(SO_4)_2.6H_2O$ [1]	0.35	This study
Optimised Whittenbury media	0.86 g $litre^{-1}$ Na_2HPO_4 0.53 g $litre^{-1}$ KH_2PO_4 3.6 μM $Fe(NH_4)_2(SO_4)_2.6H_2O$ [1]	0.31	Jahng *et al.* (1996)

[1] Fresh $Fe(NH_4)_2(SO_4)_2.6H_2O$ was added at the same time as IPTG to avoid natural oxidation of ferrous iron to ferric iron. NA, not applicable.

3.5.3 Co-expression of thioredoxin

A popular method for over-expressing recombinant proteins in a soluble form is to link the gene of interest to a second gene which is expressed well in *E. coli*. Such examples include glutathione S-transferase (Smith and Johnson, 1988) and *E. coli* thioredoxin (encoded by *trx*A) (LaVillie *et al.* 1993). The latter has a number of characteristics that make it a suitable choice for this role, including its soluble expression to 40 % of the total cell protein (LaVillie *et al.* 1993). Yasukawa *et al.* (1995) used the properties of *E. coli* thioredoxin to increase the solubility of foreign proteins in *E. coli* by the co-expression of a vector containing a *trx*A gene under the

control of a T7 promoter. This vector (pT-Trx) was used to establish if the accumulation of insoluble hydroxylase could be reduced. *E. coli* BL21(DE3) was transformed with pT7-5A (encoding Ap-resistance) and the compatible plasmid pT-Trx (encoding chloramphenicol- (Cm) resistance) and transformants were selected on LB agar containing Ap (100 $\mu g\ ml^{-1}$) and Cm (50 $\mu g\ ml^{-1}$). Co-expression of *mmoX*, *mmoY*, *mmoZ* and *trxA* was induced with IPTG for 5 h and the accumulation of inclusion bodies was investigated using phase contrast microscopy (Table 3.6). Soluble and insoluble extracts of *E. coli* BL21(DE3) [pT7-5A/pT-Trx] were prepared after induction with 0.4 mM IPTG for 5 h at 37 °C. Samples were separated by SDS-PAGE which confirmed the expression of thioredoxin (about 12 kDa) and the recombinant hydroxylase. Analysis of the insoluble extracts by SDS-PAGE (data not shown) indicated the accumulation of the α, β and γ subunits of the hydroxylase and so solubility of the hydroxylase had not been increased by co-expression of thioredoxin. Propylene oxidation enzyme assays of the soluble and insoluble extracts of *E. coli* BL21(DE3) [pT7-5A/pT-Trx] did not result in propylene oxidation activity when reconstituted with pure protein B and pure reductase from *Mc. capsulatus* (Bath).

3.5.4 Reconstitution of soluble extract with iron

A hypothesis for the lack of recombinant hydroxylase activity from *E. coli* was that the organism was not capable of assembling the di-iron centre of the α subunit of the recombinant hydroxylase correctly. If this was the case, then one would expect the iron-free apoform of the α subunit to be expressed in *E. coli* which could be particularly unstable and susceptible to degradation within the cell. Consistent with

Table 3.6: Effect of Thioredoxin and GroESL on insoluble protein accumulation in *E. coli* BL21(DE3).

Time after IPTG induction (h)	Number of cells containing inclusion bodies (%)		
	E. coli BL21(DE3) [pT7-5sMMO]	*E. coli* BL21(DE3) [pT7-5sMMO/pT-Trx]	*E. coli* BL21(DE3) [pT7-5sMMO/pT-GroE]
0	5	5	10
1	50	5	20
2	60	10	30
3	70	25	50
4	90	40	70
5	90	50	90

The control, *E. coli* BL21(DE3) [pT7-5] did not accumulate any inclusion bodies during a similar induction experiment.

this hypothesis, SDS-PAGE and Western blotting of whole cell lysates of *E. coli* BL21(DE3) [pT7-5sMMO] clearly indicated that the level of α subunit protein was present in the cell at a much lower level than the β and γ subunits (Figures 3.5 and 3.7). An attempt was made, in collaboration with Dr. P. C. Wilkins (University of Warwick, UK), to restore iron into the recombinant hydroxylase to establish if this would result in enzyme activity. In 1993, Atta *et al.* reported that the apohydroxylase of sMMO could be reconstituted by the anaerobic addition of Fe(II) followed by air oxidation, which restored 85-90 % of the original activity.

To 25 mg of *E. coli* BL21(DE3) [pT7-5sMMO] soluble extract, a 0.1-1 mM $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 1-10 mM dithiothreitol (DTT) (1:10 molar ratio) solution was incubated at room temperature for 15 min under anaerobic conditions. All anaerobic manipulations were performed under argon in septum-capped vials. Solutions were made anaerobic by several cycles of evacuation and flushing with argon. Air was then added to the reaction mixture. 4-20 nmol of pure protein B and reductase were added and propylene oxidation assays performed at 30, 37 and 45 °C but no enzyme activity was detected. This experiment was repeated with insoluble extract and crude cell extract, but the same lack of activity was found.

3.5.5 Co-expression of GroESL

When using a T7-promoter to over-express a protein in *E. coli* a higher level of expression of the *E. coli* chaperone GroESL may be needed to ensure proper folding of the foreign proteins. Co-expression of the GroESL operon (under the control of a T7 promoter) by Yasukawa *et al.* (1995) improved the solubility of four out of eight eukaryotic proteins tested in *E. coli*. This vector (pT-GroE) was used to establish if co-expression of GroESL would improve the solubility of the recombinant hydroxylase. *E. coli* BL21(DE3) was transformed with pT7-5A (encoding Ap-resistance) and the compatible plasmid pT-GroE (encoding Cm-resistance) and transformants were selected on LB agar containing Ap (100 $\mu g\ ml^{-1}$) and Cm (50 $\mu g\ ml^{-1}$). Co-expression of *mmoX, mmoY, mmoZ* and *groESL* was induced with 0.4 mM IPTG for 5 h at 37 °C and the accumulation of inclusion bodies was investigated using phase contrast microscopy (Table 3.6). The results suggested that GroESL did not improve the solubility of the hydroxylase protein in *E. coli*

BL21(DE3). In addition, SDS-PAGE of whole cell lysates confirmed that greater than 90 % of the recombinant hydroxylase was found in the insoluble fraction and propylene oxidation assays confirmed the lack of functional hydroxylase expression.

3.5.6 Activation of recombinant hydroxylase with hydrogen peroxide

Hydrogen peroxide (H_2O_2) can replace protein B, the reductase, NADH and oxygen in the activation of sMMO from *Mc. capsulatus* (Bath) (Jiang *et al.*, 1993). Therefore, the hydroxylase and H_2O_2 can be used to obtain catalysis of oxidation of methane and other sMMO substrates in a reaction known as the peroxide shunt. It has been shown that the α subunit of the hydroxylase contains the substrate binding site (Prior and Dalton, 1985; Nordlund *et al.*, 1992; Rosenzweig *et al.*, 1993; George *et al.*, 1996) and that the complete sMMO enzyme system (i.e. the hydroxylase, protein B and the reductase) is required for enzyme activity using oxygen as the electron acceptor (Dalton, 1991). To establish if the recombinant hydroxylase could be activated by H_2O_2, propylene oxidation assays using H_2O_2 as the oxidant were attempted.

Soluble extracts of *E. coli* BL21(DE3) [pT7-5A] were prepared. Due to the low level of α subunit protein expression observed, it was deemed necessary to prepare concentrated protein extracts to maximise the chance of assaying active protein. Soluble extracts at a protein concentration of 40 mg ml^{-1} were prepared and it was estimated from SDS-PAGE of whole cell lysates that approximately 2-3 mg would constitute the total recombinant hydroxylase. Propylene oxidation assays were performed with 100-200 mM H_2O_2 as oxidant with and without 5-10 μM $Fe(NH_4)_2(SO_4)_2.6H_2O$, but no enzyme activity was detected. Also, reconstitution of

the soluble extract with iron (as described in Section 3.5.4) did not result in enzyme activity. These experiments were repeated with insoluble extracts (protein concentration of 18 mg ml^{-1}) of *E. coli* BL21(DE3) [pT7-5A] but no enzyme activity was detected.

3.6 Effect of copper on sMMO expression in *E. coli* BL21(DE3)

To induce expression of sMMO in methanotrophs that possess both pMMO and sMMO, the organisms have to be grown in conditions of low copper (Stanley *et al.*, 1983). In batch culture this is normally achieved by acid washing flasks to deplete copper and by using Chelex-treated water to remove all metals (Tsien *et al.*, 1989; Martin, 1994). It has been established that copper can cause the loss of prosthetic groups from the reductase component of sMMO from *Mc. capsulatus* (Bath) *in vitro* (Green *et al.*, 1985). Copper also represses the transcription of sMMO genes (Nielsen *et al.*, 1996, 1997). Copper could have a deleterious effect on hydroxylase expression in *E. coli* and this was investigated *via* the use of copper-free M9 minimal medium and optimised Whittenbury medium. *Ms. trichosporium* OB3b was routinely grown in the same batch of copper-free glassware and water used to prepare the growth medium and sMMO expression was observed in batch culture. This confirmed that enough copper had been depleted to derepress sMMO transcription.

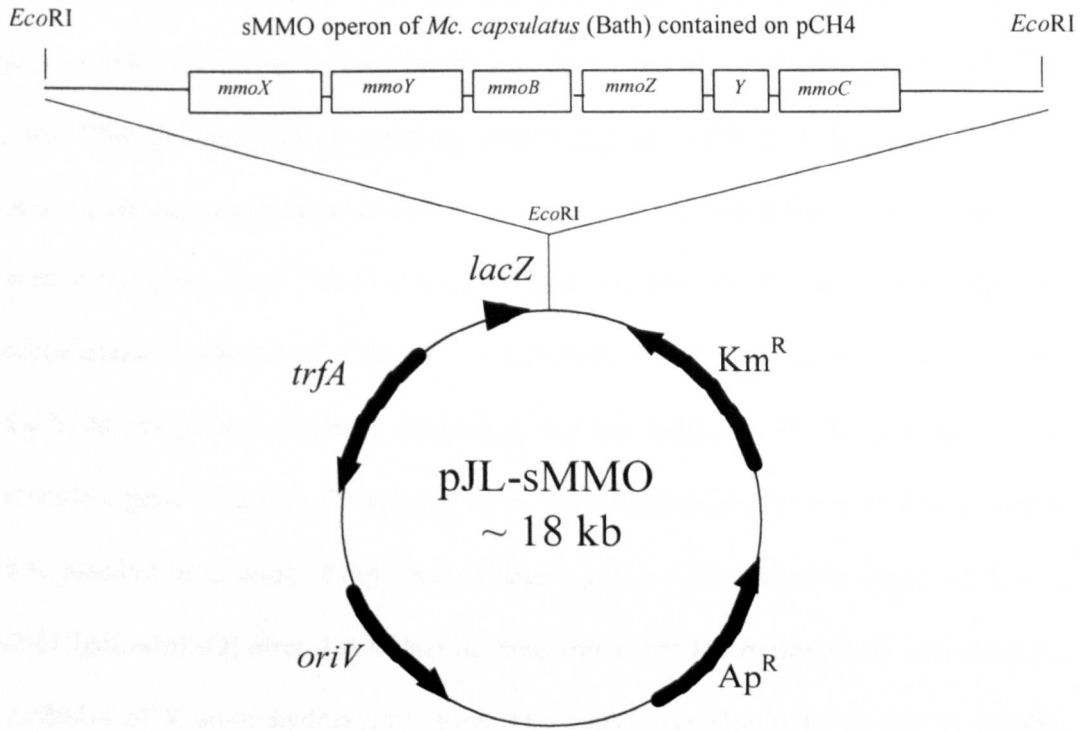
Copper depleted medium had no effect upon the growth rate of *E. coli* compared to the same medium prepared in the usual way. Expression of *E. coli* BL21(DE3) [pT7-5sMMO] was induced with 0.4 mM IPTG at 37 °C for 5 h and the soluble and insoluble extracts were analysed by SDS-PAGE and Western blotting with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath). Expression

levels were no different in copper-free medium when compared to medium made without removing copper. Propylene oxidation assays confirmed that the hydroxylase was inactive. Addition of 5 μM $Fe(NH_4)_2(SO_4)_2$ to the copper-free growth medium also had no effect.

3.7 Effect of upstream DNA sequences on sMMO expression in *E. coli*

Upstream activator sequences located more than 100 bp from the transcription start site have been found to be required for the expression of nitrogen fixation genes in *Klebsiella pneumoniae* (Buck *et al.*, 1986). To determine if there were sequences flanking the sMMO operon that were required for the functional expression of sMMO, a 12 kb *Eco*RI fragment was subcloned from pCH4 (Stainthorpe *et al.*, 1990) into the broad host range plasmid, pJB3Km1 (Blatny *et al.*, 1997). This fragment contained the entire sMMO operon and about 6 kb of additional sequence: 3 kb upstream and 3 kb downstream of the sMMO operon. 20 transformants of *E. coli* DH1 that contained an insert were restriction mapped. After digestion with *Bam*HI, *Bgl*II and *Sph*I, one clone was identified that contained the 12 kb insert in the correct orientation with respect to the *lac* promoter. The plasmid was designated pJL-sMMO (Figure 3.8). *E. coli* DH1 [pJL-sMMO] was grown in LB medium and expression of the sMMO operon was induced at an OD_{540} of 0.6 with 1 mM IPTG at 37 °C. The OD_{540} was monitored during induction and unlike the decrease in growth rate that was observed after IPTG induction of *E. coli* BL21(DE3) [pT7-5A] and [pT7-5sMMO] (Section 3.4), no comparable difference in growth rate was seen between *E. coli* DH1 [pJL-sMMO] and the control, *E. coli* DH1 [pJB3Km1]. This was attributed to the

Figure 3.8: Construction of pJL-sMMO. A 12 kb *Eco*RI fragment was subcloned from pCH4 into pJB3Km1.



trfA, gene encoding an essential initiator protein

oriV, origin of replication

Ap^R, gene encoding ampicillin resistance

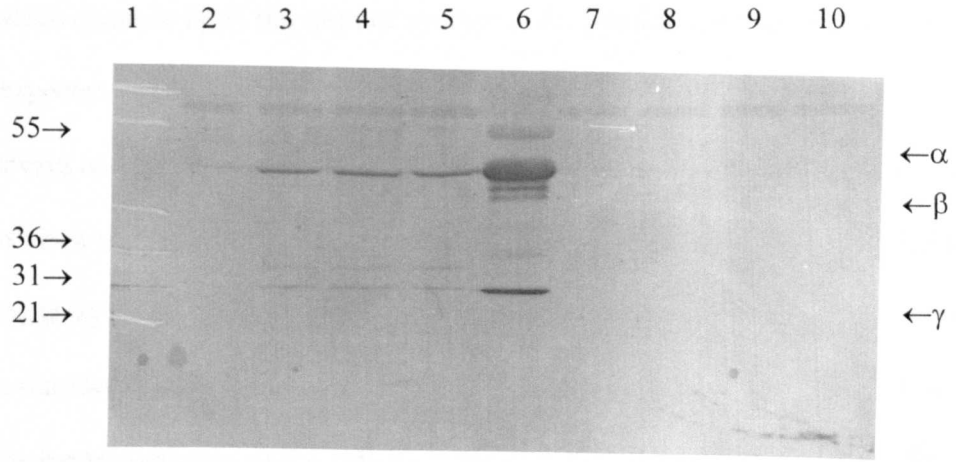
Km^R, gene encoding kanamycin resistance

lower level of expression from a *lac* promoter in comparison to a T7 promoter. Whole cell lysates were separated by SDS-PAGE which indicated that there was no detectable difference between *E. coli* DH1 [pJB3Km1] and *E. coli* DH1 [pJLsMMO] using Coomassie Brilliant Blue. The SDS-PAGE gel was then Western blotted and probed with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) (Figure 3.9). This illustrated cross-reactivity only in extracts of *E. coli* DH1 [pJL-sMMO], thus confirming expression from the *lac* promoter. A low level of expression was seen at 0 h (less than 1 % of the total cell protein) and after 4 h the hydroxylase had accumulated to about 5 % of the total cell protein. Poor expression of the α subunit of the hydroxylase was observed, suggesting that the additional DNA sequence did not include a gene necessary for activity of the hydroxylase (or if it was included, then it was inactive in *E. coli*). Propylene oxidation assays of the soluble extract of *E. coli* DH1 [pJL-sMMO] after 4 h induction confirmed that the hydroxylase was inactive. Addition of 8 nmol hydroxylase from *Mc. capsulatus* (Bath) to 15 mg of soluble extract, resulted in a specific activity of 3 ± 0.5 nmol min^{-1} mg $protein^{-1}$. No enzyme activity was observed for *E. coli* DH1 [pJB3Km1].

3.8 Discussion

The work presented in this Chapter has illustrated that sMMO from *Mc. capsulatus* (Bath) was inactive when expressed in *E. coli* due to the expression of inactive hydroxylase. Enzyme activity was detected for recombinant protein B and reductase which was in agreement with the results of West *et al.* (1992). The reason why the hydroxylase was inactive in *E. coli* is unclear but is probably due to a number of factors.

Figure 3.9: Western blot of hydroxylase expression in *E. coli* DH1 [pJL-sMMO]. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2 to 5, whole cell lysates of *E. coli* DH1 [pJL-sMMO] 0, 1, 2 and 4 h respectively after induction; lane 6, 10 μg pure hydroxylase from *Mc. capsulatus* (Bath); lanes 7 to 10, whole cell lysates of *E. coli* DH1 [pJB3Km1] 0, 1, 2 and 4 h respectively after induction. Anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) was used as the probe.



Inducing the expression of sMMO in *E. coli* resulted in inclusion body formation. Evidence has suggested that in general, there is no relationship between inclusion body formation and: (1) the type of promoter used; (2) the formation of disulphide bonds; (3) protein molecular mass; (4) level of expression in the cell (Kane and Hartley, 1988). The model of inclusion body formation in *E. coli* proposed by Kane and Hartley (1988) suggested that there were three fates for the recombinant protein following transcription and translation: (1) the conformation of the protein is normal and the protein is stable and soluble; (2) the protein is soluble but retains an abnormal conformation and would be degraded by proteases; (3) the protein retains a conformation that is protease resistant and leads to inclusion body formation, the rate of which depends upon the rates of synthesis, degradation and aggregation. It would be expected that some recombinant proteins would follow a combination of these pathways and this could be considered for the expression of sMMO in *E. coli*. *In vitro* translation of total cell RNA of *Mc. capsulatus* (Bath) has shown that the subunits of the hydroxylase at least, are synthesised as their native molecular mass values and that post-translational modifications do not occur (Green and Dalton, 1988). Therefore, following transcription of the sMMO genes in *E. coli*, it could be postulated that the β and γ subunits of the hydroxylase, protein B and the reductase were expressed as soluble proteins but, as their expression levels increased, they aggregated to form protease resistant inclusion bodies. In contrast, the α subunit of the hydroxylase may have been expressed as soluble protein that adopted a protease-sensitive conformation and was degraded by the cell. This could explain why SDS-PAGE of whole cell lysates always indicated low levels of α subunit expression in *E. coli*.

Another reason for the poor expression of *mmoX* in *E. coli* could be due to a Shine-Dalgarno sequence that was poorly recognised by *E. coli* and so the mRNA would be poorly translated. The Shine-Dalgarno consensus sequence of *E. coli* is 5'...AGGAGG...3' and analysis of mRNA sequences upstream of each gene of the sMMO operon from *Mc. capsulatus* (Bath) can be used to predict a likely Shine-Dalgarno sequence for each open reading frame (ORF) (Figure 3.10). The predicted

Figure 3.10: Predicted Shine-Dalgarno sequence for each ORF of the sMMO operon mRNA from *Mc. capsulatus* (Bath). Predicted Shine-Dalgarno sequence in **bold**; initiation codon underlined. *E. coli* consensus is 5'...AGGAGG...3'.

5′ UUA**CGGAGG**AAACAAGUAAUG 3′ *mmoX*

5′ CCA**AAAAGG**AACAUCGAUAUG 3′ *mmoY*

5′ AA**UAGAGG**AACUAUUACGAUG 3′ *mmoB*

5′ ACGCUUA**AGGAGA**AUGACAUG 3′ *mmoZ*

5′ CUUUCGUA**AGGAAA**UCACCAUG 3′ *orfY*

5′ CAUUG**AACAGG**UAAGUUUAUG 3′ *mmoC*

Shine-Dalgarno sequence for *mmoX* is very similar to the *E. coli* consensus and so poor translation of mRNA would not appear to be a problem.

To study more precisely the expression of the hydroxylase component in *E. coli*, pT7-5A was used. This plasmid contained only the *mmoX*, *mmoY* and *mmoZ* genes under the control of a T7 promoter. It might be predicted that the assembly of the hydroxylase protein would be disrupted using pT7-5A because *mmoB*, *mmoC* and

orfY were no longer present. It could be postulated that translation of the fully functional sMMO in *Mc. capsulatus* (Bath) occurs in such a way that the entire sMMO operon must be transcribed and translated for the correct folding of the protein. However, since no enzyme activity had been obtained by expressing the whole sMMO operon in *E. coli*, attempts were made to try to understand why the hydroxylase in particular was inactive. Analysis of pT7-5A expression in *E. coli* showed the same problem of poor α subunit expression and large numbers of inclusion bodies. Consequently, the hydroxylase protein was largely insoluble in *E. coli* and this could not be overcome using any of the methods tested.

Another reason for the lack of sMMO activity could be due to the incorrect assembly of the hydroxylase. The α subunits form the active site of the enzyme and contain a μ-oxo bridged di-iron centre (Fox *et al.*, 1988; Rosenzweig *et al.*, 1993). One of the major problems with using *E. coli* as a heterologous host is that proteins with prosthetic groups are often expressed as inactive proteins (Weickert *et al.*, 1996). To expedite the formation of the di-iron centre, ferrous ammonium sulphate was added to the culture medium but this did not improve the expression levels of the hydroxylase or result in active protein. In an attempt to obtain functional expression of sMMO from *Ms. trichosporium* OB3b in *E. coli*, Jahng *et al.* (1996) co-expressed flavin reductase to increase the intracellular concentration of ferrous iron. Flavin reductase mobilises iron from ferrisiderophores by reducing insoluble Fe(III) to soluble Fe(II). Simultaneous expression of sMMO and flavin reductase did not result in enzyme activity, suggesting that low activity of the recombinant hydroxylase was probably not due to low levels of iron (Jahng *et al.*, 1996). In addition, the structure of the active site of sMMO is closely related to ribonucleotide reductase (Nordlund *et*

al., 1992). Ribonucleotide reductase plays a central role in DNA biosynthesis (Stubbe, 1990) and is therefore ubiquitous in nature. Its existence in *E. coli* suggests that this organism almost certainly has the capacity to assemble the di-iron centre of sMMO, since the principal ligands are similar to those in the R2 subunit of ribonucleotide reductase. This suggests that the inactive hydroxylase expression in *E. coli* was not due to a problem with assembly of the di-iron active site of sMMO.

There are numerous reports of the co-expression of molecular chaperones resulting in correctly folded and active recombinant proteins in *E. coli* (reviewed by Hockney, 1994; Cole, 1996; Weickert *et al.*, 1996). GroESL from *E. coli* has been characterised both structurally and mechanistically (Braig *et al.*, 1994). It has been shown that proteins in their partially folded form are prevented from forming inclusion bodies by insertion into GroEL. Furthermore, GroES binding to the complex formed between GroEL and the target protein is also thought to facilitate target protein release. The proteins released from GroEL mature to fully folded forms or become re-bound. The effect of the GroESL molecular chaperone of *E. coli* was investigated during expression of recombinant sMMO, but there was no improvement in solubility or assembly. Reasons for this are unknown, but there are many other examples in which molecular chaperones have also had no effect on expression of other recombinant proteins (Weickert *et al.*, 1996). This does raise the question, however, of whether a molecular chaperone specific to methanotrophs could be required for functional expression of sMMO. DNA sequencing 400 bp upstream of *mmoX* of *Ms. trichosporium* OB3b has revealed a gene encoding a protein with 49 % amino acid identity to GroESL of *Bacillus stearothermophilus* and *Neisseria gonorrhoeae* (I. R. McDonald and J. C. Murrell, unpublished). No such homologue

has yet been identified in *Mc. capsulatus* (Bath). It has been shown that a number of different proteins require GroESL for correct assembly of native proteins (reviewed by Ryalls *et al.*, 1991). For example, ribulose 1,5-bisphosphate carboxylase (Rubisco) from the purple bacterium *Chromatium vinosum* has an absolute requirement for homologous or heterologous (*E. coli*) GroESL for correct assembly (Dionisi *et al.*, 1996). Suitable experiments to determine whether GroESL was required for the correct folding of sMMO would be: (1) the co-expression of *smmo* and *groESL* from *Ms. trichosporium* OB3b in *E. coli*; (2) to determine if inactivation of *groESL* (by the insertion of a Km-resistance cassette) had a deleterious effect upon sMMO activity in *Ms. trichosporium* OB3b.

E. coli is a good starting point for the heterologous expression of proteins but it is not always capable of expressing active protein (McKay *et al.*, 1997). Although the work presented in this Chapter has not defined exactly why sMMO is inactive in *E. coli*, it was considered necessary to express the sMMO genes in other hosts (see Chapters 4, 7 and 8) in an attempt to obtain functional expression of sMMO.

CHAPTER 4

Heterologous expression of sMMO from: (1) *Methylosinus trichosporium* OB3b in *Escherichia coli* and *Pseudomonas putida*; (2) *Methylococcus capsulatus* (Bath) in *P. putida*

4.1 Introduction

In 1994, Jahng and Wood reported the heterologous expression of sMMO from *Ms. trichosporium* OB3b in *P. putida* F1. The sMMO genes were subcloned into a broad host range vector and the resulting construct (pSMMO20) was electroporated into *P. putida* F1. SDS-PAGE and Western blotting were used to confirm the expression of sMMO polypeptides. Since sMMO can oxidise TCE, Jahng and Wood (1994) used the degradation of TCE as an assay to detect for recombinant sMMO enzyme activity. Using *P. putida* F1 [pSMMO20], the maximum rate of TCE degradation was 4.9-5.3 nmol of TCE min^{-1} mg $protein^{-1}$, compared to 40 nmol of TCE min^{-1} mg $protein^{-1}$ for *Ms. trichosporium* OB3b. Although similar levels of sMMO expression were observed (as judged by SDS-PAGE and Western blotting), TCE was oxidised at a rate 8-fold less in the recombinant organism. Although *P. putida* F1 contains toluene dioxygenase (TDO) that will degrade TCE at a rate of 1.8 nmol min^{-1} mg $protein^{-1}$ (Wackett and Gibson, 1988) expression of TDO was only induced by the presence of toluene in the growth medium. *P. putida* F1 grown in the absence of toluene degraded 6 % of 20 μM TCE after 5 h in comparison to *P. putida* F1 [pSMMO20] which degraded 42 % of 10 μM TCE after 5 h at a degradation rate of 5 nmol min^{-1} mg $protein^{-1}$. *Ms. trichosporium* OB3b expressing sMMO degraded 100 % of 80 μM TCE in 20 min at a rate of 40 nmol min^{-1} mg $protein^{-1}$ (Tsien *et al.*, 1989) at the same cell density as *P. putida* F1 [pSMMO20]. This comparison with *Ms. trichosporium* OB3b suggested that the activity of the recombinant sMMO was poor. As an additional test for the expression of functional sMMO, the ability of *P. putida* F1[pSMMO20] to degrade chloroform was tested. sMMO will oxidise chloroform (Alvarez-Cohen and McCarty, 1991; Alvarez-Cohen *et al.*, 1992) but

wild-type *P. putida* F1 will not metabolise this compound. It was shown that 25 % of 20 μM chloroform was degraded in 5 h by *P. putida* F1 [pSMMO20] but not by wild-type *P. putida* F1.

To quantify sMMO activity, the propylene oxidation assay has been routinely used (for example Fox *et al.*, 1989; Pilkington and Dalton, 1990). Jahng *et al.* (1996) reported that cell free extracts of *P. putida* F1 [pSMMO20] did not oxidise propylene after 18 min incubation, although propylene oxide production was observed after 90 min incubation. This provided additional evidence that the recombinant sMMO was largely inactive. Addition of pure hydroxylase from *Ms. trichosporium* OB3b to soluble extracts resulted in an enzyme activity of 29 nmol min^{-1} mg $hydroxylase^{-1}$ (compared to an enzyme activity of 324 nmol min^{-1} mg $hydroxylase^{-1}$ for pure *Ms. trichosporium* OB3b sMMO proteins) (Jahng *et al.*, 1996). This indicated that the hydroxylase component was inactive and that protein B and the reductase were functionally expressed. West *et al.* (1992) reported that the hydroxylase from *Mc. capsulatus* (Bath) was inactive when expressed in *E. coli* and it appeared that the same scenario occurred when sMMO from *Ms. trichosporium* OB3b was expressed in *P. putida* F1.

In addition to the negligible amount of recombinant sMMO activity observed, another problem with the *P. putida* expression system of Jahng and Wood (1994) was that only 15 % of the 20 or so shake flask cultures inoculated with *P. putida* F1 [pSMMO20], degraded appreciable levels of TCE. The most active cultures degraded 35 % of 20 μM TCE in 5 h, where as the remaining 85 % of cultures showed about 15 % degradation (negative controls degraded 6 % in 5 h). SDS-PAGE confirmed

that sMMO was expressed in both active and inactive phenotypes of *P. putida* F1 [pSMMO20].

From the work of Jahng and Wood (1994) and Jahng *et al.* (1996) there was some evidence for the functional expression of sMMO in *P. putida* F1, albeit with poor activity. Therefore, the aim of the work reported in this Chapter was to repeat the methods used by Jahng and Wood (1994) in an attempt to obtain consistent recombinant *Ms. trichosporium* OB3b sMMO activity. In addition, a broad host range vector containing the sMMO genes from *Mc. capsulatus* (Bath) was constructed that could be transformed into *P. putida*, to elucidate if the previously observed lack of enzyme activity in *E. coli* (Chapter 3) could be overcome by using a different heterologous host.

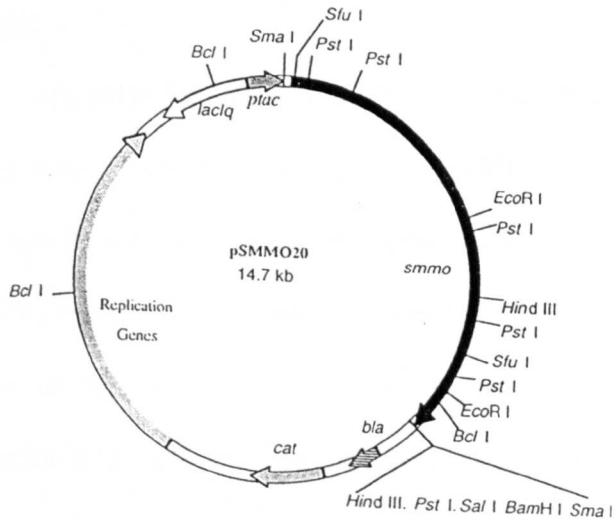
4.2 Expression of sMMO from *Ms. trichosporium* OB3b in *E. coli* and *P. putida*

4.2.1 Transformation of bacterial strains for expression studies

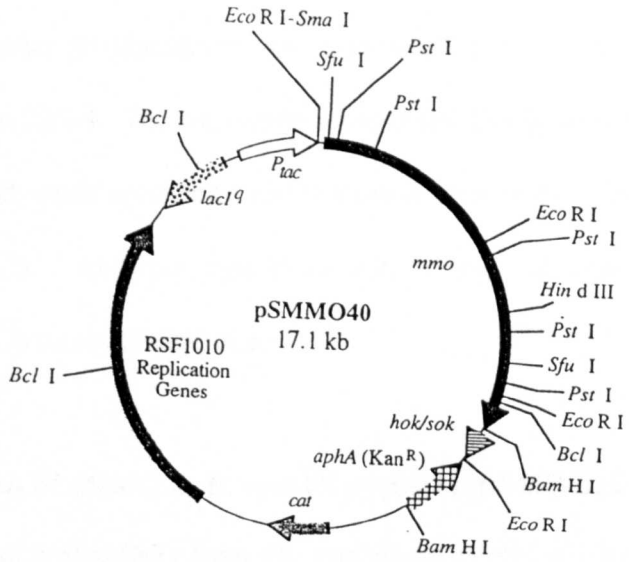
P. putida F1, pSMMO20 and pSMMO40 (Figure 4.1) were provided by Dr. T. Wood (University of California, USA). The integrity of the plasmids were determined by digestion with *Eco*RI, *Hin*dIII, *Pst*I and *Sma*I. *E. coli* BL21(DE3) and *P. putida* F1 were transformed with pSMMO20 and pSMMO40. *E. coli* BL21(DE3) and *P. putida* NCIMB 11767 were transformed with pJL-sMMO. Attempts to transform *P. putida* NCIMB 8859, *P. putida* NCIMB 10007 and *P. putida* NCIMB 8248 with pSMMO20 and pJL-sMMO were unsuccessful. All transformations were performed by electroporation as described in Section 2.6.2. The negative controls used in recombinant sMMO activity measurements were *E. coli* BL21(DE3)

Figure 4.1: Restriction maps of (a) pSMMO20 and (b) pSMMO40. Reproduced from Jahng and Wood (1994) and Jahng *et al.* (1996) respectively.

(a)



(b)



Key: *bla*, β-lactamase gene; *cat*, Cm acetyl-transferase gene; *ptac*, *tac* promoter; *lacI*^{q}, gene encoding the repressor protein of the β-galactosidase operon; *hok/sok*, genes encoding the Hok/Sok plasmid stabilisation system (Gerdes, 1988).

[pJB3Km1] and *P. putida* NCIMB 11767 [pJB3Km1].

4.2.2 Preparation of anti-serum against the hydroxylase from *Ms. trichosporium* OB3b

Anti-sera against the sMMO proteins from *Mc. capsulatus* (Bath) do not cross-react with sMMO proteins from *Ms. trichosporium* OB3b (C. West, personal communication). Therefore, anti-serum against the hydroxylase of *Ms. trichosporium* OB3b was prepared. The hydroxylase was purified from *Ms. trichosporium* OB3b by R. Titmus (University of Warwick, UK) in the same way as described for *Mc. capsulatus* (Bath) (Section 2.12.1). Due to the problem encountered with the immune serum obtained against the hydroxylase from *Mc. capsulatus* (Bath) (Section 3.3), the purified hydroxylase from *Ms. trichosporium* OB3b was denatured by heating at 80 °C for 5 min, prior to injection. Anti-sera were then raised and prepared as described in Section 2.12.4. The anti-serum cross-reacted with the β and γ subunits of the hydroxylase with equal specificity and showed weaker cross-reactivity with the α subunit (Figure 4.2). Anti-sera against protein B and the reductase from *Ms. trichosporium* OB3b were not available.

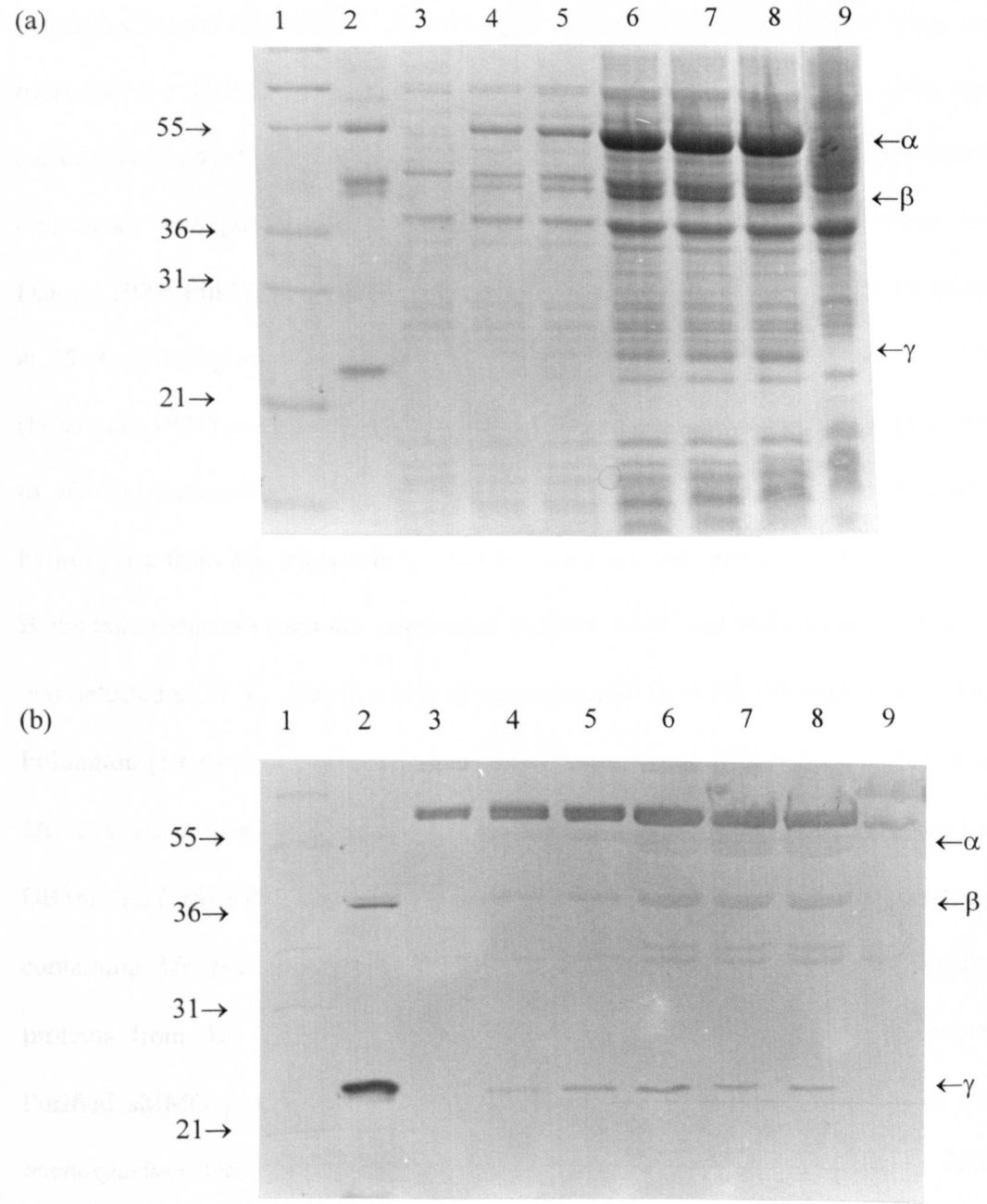
4.2.3 Expression of sMMO in *E. coli* BL21(DE3) [pSMMO20]

The expression of sMMO from *Mc. capsulatus* (Bath) (Chapter 3) concluded that the hydroxylase was inactive when expressed in *E. coli*. To compare these results with those of sMMO from *Ms. trichosporium* OB3b expressed in *E. coli*, pSMMO20 was electroporated into *E. coli* BL21(DE3). Jahng *et al.* (1996) reported that *E. coli*

BL21(DE3) [pSMMO20] did not give rise to sMMO activity, but the reason for the inactivity of recombinant sMMO was not reported.

E. coli BL21(DE3) was electroporated with pSMMO20 and transformants were selected on LB agar containing Cm (50 μg ml^{-1}) (no transformants were obtained after plating *E. coli* BL21(DE3) onto LB agar containing Cm (50 μg ml^{-1})). Transformants were grown at 30 °C to an OD_{540} of 0.5 and expression was induced with 1 mM IPTG. After 5 h induction, greater than 90 % of the cells contained inclusion bodies. Aliquots were sampled at 0 to 5 h after induction, boiled, separated by SDS-PAGE and then Western blotted with anti-serum against the hydroxylase from *Ms. trichosporium* OB3b (Figure 4.2). Results confirmed that expression of the α, β and γ subunits of the hydroxylase had occurred. Analysis of the soluble and insoluble extracts indicated that all of the recombinant hydroxylase was identified in the insoluble fraction (data not shown). To optimise the growth conditions for the expression of soluble protein, the growth temperature was varied between 20 and 30 °C and the IPTG concentration varied between 0.1 and 1 mM. Consistent with the results obtained for the expression of the recombinant hydroxylase from *Mc. capsulatus* (Bath) in *E. coli* using this method (Section 3.5), this strategy did not result in the expression of soluble hydroxylase. Whole cell lysates of *E. coli* BL21(DE3) [pSMMO20] and soluble extracts of *Ms. trichosporium* OB3b expressing sMMO probed with anti-sera against protein B and the reductase of *Mc. capsulatus* (Bath) did not reveal any cross-reactivity. This confirmed the observations of Dr. C. West (University of Warwick, UK) that anti-sera against the sMMO components from *Mc. capsulatus* (Bath) do not cross-react with *Ms. trichosporium* OB3b sMMO proteins. One of the major difficulties encountered with this work was that pure

Figure 4.2: (a) SDS-PAGE and (b) Western blotting of sMMO expression from *Ms. trichosporium* OB3b in *E. coli* BL21(DE3). (a) Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, 5 μg of pure hydroxylase from *Ms. trichosporium* OB3b; lanes 3 to 5, whole cell lysates (approximately 15 μg) of *E. coli* BL21(DE3) [pSMMO20] at 0, 1, and 2 h respectively after induction with IPTG; lanes 6 to 8, whole cell lysates (approximately 30 μg) of *E. coli* BL21(DE3) [pSMMO20] at 3, 4 and 5 h respectively after induction with IPTG; lane 9, approximately 30 μg of whole cell lysate of *E. coli* BL21(DE3). (b) Western blot of lanes 2 to 9 probed with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b.


(a)
1 2 3 4 5 6 7 8 9
55→
36→
31→
21→
←α
←β
←γ
(b)
1 2 3 4 5 6 7 8 9
55→
36→
31→
21→
←α
←β
←γ

proteins from *Ms. trichosporium* OB3b were not available to complement soluble extracts of *E. coli* BL21(DE3) [pSMMO20]. (All of the pure hydroxylase from *Ms. trichosporium* OB3b had been used to raise anti-serum). Therefore, pure *Mc. capsulatus* (Bath) sMMO proteins were used, which have been shown to functionally cross-react with pure sMMO proteins of *Ms. trichosporium* OB3b (Stirling and Dalton, 1979; Pilkington, 1986). Optimal growth of *Mc. capsulatus* (Bath) occurred at 45 °C (Pilkington and Dalton, 1990) and at 30 °C for *Ms. trichosporium* OB3b (Fox *et al.*, 1991) and so enzyme assays are routinely performed at these temperatures to obtain maximum sMMO activity. Pilkington (1986) established that pure hydroxylase from *Ms. trichosporium* OB3b gave functional activity with pure protein B and pure reductase from *Mc. capsulatus* (Bath) at 45 °C and that no enzyme activity was detected at 30 °C. Due to a lack of pure reductase from *Ms. trichosporium* OB3b, Pilkington (1986) could not determine the enzyme activity of pure hydroxylase from *Mc. capsulatus* (Bath) with pure protein B and reductase from *Ms. trichosporium* OB3b. To further determine the optimum temperature for assaying soluble extracts containing *Ms. trichosporium* OB3b sMMO proteins complemented with purified proteins from *Mc. capsulatus* (Bath), the following experiment was performed. Purified sMMO proteins from *Mc. capsulatus* (Bath) and soluble extracts of *Ms. trichosporium* OB3b expressing sMMO were assayed at 45, 37 and 30 °C. Soluble extracts of *Ms. trichosporium* OB3b were then complemented *in vitro* with pure sMMO proteins from *Mc. capsulatus* (Bath) (Table 4.1). The maximum sMMO enzyme activity for purified proteins from *Mc. capsulatus* (Bath) and soluble extracts from *Ms. trichosporium* OB3b under the conditions tested was at 45 °C. Addition of pure hydroxylase from *Mc. capsulatus* (Bath) to *Ms. trichosporium* OB3b soluble

Table 4.1: Cross-reactivity of sMMO components from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b at different temperatures.

Assay temperature (°C)	Protein	Specific activity (nmol min^{-1} mg $protein^{-1}$)
45	*M. caps* A + B + C	131 ± 24
	OB3b soluble extract	25 ± 4
	OB3b soluble extract + A	27 ± 3
	OB3b soluble extract + B	17 ± 3
	OB3b soluble extract + C	64 ± 7
37	*M. caps* A + B + C	79 ± 11
	OB3b soluble extract	23 ± 3
	OB3b soluble extract + A	24 ± 1
	OB3b soluble extract + B	25 ± 1
	OB3b soluble extract + C	45 ± 5
30	*M. caps* A + B + C	50 ± 10
	OB3b soluble extract	22 ± 5
	OB3b soluble extract + A	17 ± 1
	OB3b soluble extract + B	38 ± 4
	OB3b soluble extract + C	33 ± 2

M. caps A + B + C, 8 nmol each of pure proteins A (hydroxylase), B (protein B) and C (reductase) from *Mc. capsulatus* (Bath); OB3b soluble extract, 9 mg of soluble extract of *Ms. trichosporium* OB3b expressing sMMO. 16 nmol each of pure proteins A, B and C were added to OB3b soluble extracts. Assays were performed in 500 µl 25 mM MOPS, pH 7.0 with 481 nmol of propylene and 224 µmol NADH in each assay. The GC retention time of propylene oxide was 1.81-1.87 min in all assays. Each result was the mean of three experiments.

extracts had a negligible effect on enzyme activity but the highest specific activity was recorded at 45 °C. Addition of pure protein B from *Mc. capsulatus* (Bath) to soluble extracts of *Ms. trichosporium* OB3b increased the specific activity by almost two-fold at 30 °C, but at 45 °C inhibition of sMMO activity was observed. Addition

of pure *Mc. capsulatus* (Bath) reductase caused a three-fold increase in enzyme activity at 45 °C and also increased the enzyme activity at 30 °C, but to a lesser extent. These results are not definitive because poor (or lack of) functional cross-reactivity could be attributed to the concentration of pure *Mc. capsulatus* (Bath) sMMO component used, not being rate-limiting in soluble extracts of *Ms. trichosporium* OB3b. However, assays to determine the cross-reactivity of recombinant *Ms. trichosporium* OB3b soluble extracts with pure proteins from *Mc. capsulatus* (Bath) were performed at 45 °C. Due to the lack of cross-reactivity of soluble extracts observed with protein B at 45 °C, enzyme assays were also performed at 30 °C.

Propylene oxidation enzyme assays were performed with soluble extracts of *E. coli* BL21(DE3) [pSMMO20] (expressing recombinant sMMO from *Ms. trichosporium* OB3b) after growth at 30 °C with 1 mM IPTG for 5 h. All enzyme assays were performed within 90 min of harvesting the cells (Table 4.2). No enzyme activity was detected in soluble extracts of *E. coli* BL21(DE3) [pSMMO20] but the addition of pure hydroxylase from *Mc. capsulatus* (Bath) did result in sMMO activity. The hydroxylase has an intrinsic requirement for iron (Fox *et al.*, 1988; Green and Dalton, 1988) but the addition of 4 μM $Fe(NH_4)_2(SO_4)_2.6H_2O$ to the growth medium at the same time as IPTG did not result in enzyme activity in the soluble extracts.

4.2.4 Expression of sMMO in *P. putida* F1 [pSMMO20]

pSMMO20 encoded Cm-resistance and so to confirm that *P. putida* F1 was sensitive to Cm, a dilution series of wild-type *P. putida* F1 was streaked on to LB agar containing Cm (50-600 μg ml^{-1}). Growth was not observed on LB agar containing

Table 4.2: Complementation of soluble extracts of *E. coli* BL21(DE3) [pSMMO20] with purified sMMO proteins from *Mc. capsulatus* (Bath).

Protein	Specific activity (nmol min^{-1} mg $protein^{-1}$)
A + B + C	128 ± 8
OB3b soluble extract	29 ± 6
Soluble extract of *E. coli* BL21(DE3)	0
Soluble extract [pSMMO20]	0[a]
Soluble extract [pSMMO20] + B + C	0
Soluble extract [pSMMO20] + A + C	7.0 ± 0.9
Soluble extract [pSMMO20] + A + B	1.4 ± 0.2
Soluble extract [pSMMO20] + A	2.3 ± 0.1
Soluble extract [pSMMO20] + B	0[b]
Soluble extract [pSMMO20] + C	0[b]
A + B	0[b]
A + C	0[b]
B + C	0

[a] No activity was detected after 90 min incubation. [b] Assays also performed at 30 °C with the same result. Purified proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase; OB3b soluble extract, 9 mg of soluble extract of *Ms. trichosporium* OB3b expressing sMMO; Soluble extract (pSMMO20), 8 mg of soluble extract of *E. coli* BL21(DE3) [pSMMO20]. Assays were performed at 45 °C in 500 µl 25 mM MOPS, pH 7.0. 8 nmol of proteins A, B and C, 481 nmol of propylene and 224 µmol NADH were used. The GC retention time of propylene oxide was 1.82 min in all assays. The results are the mean of three experiments.

500 and 600 µg ml^{-1} of Cm after incubation for 7 days at 30 °C, confirming that these concentrations were appropriate for selection of antibiotic resistant colonies. *P. putida* F1 was electroporated with pSMMO20 and transformed cells were streaked on to LB agar containing Cm (500 µg ml^{-1}) and incubated for 2-3 days at 30 °C. For liquid

cultures, cells were grown in LB. Alternatively, the two stage growth method of Jahng and Wood (1994) was used in an attempt to overcome slow growth in M9 medium. This was achieved as follows. 20 shake flask cultures were made by inoculating a single colony into 20 ml of LB containing Cm (500 $\mu g\ ml^{-1}$) and incubated overnight. For each of these precultures, 0.5 ml of the LB culture was washed twice with M9 medium and transferred to 50 ml of M9 medium. μ of *P. putida* F1 [pSMMO20] was 0.1 to 0.15 h^{-1} (compared to 0.2 to 0.3 h^{-1} reported by Jahng and Wood (1994) in M9 medium and 0.35 h^{-1} in LB. μ of wild-type *P. putida* was 0.45 h^{-1} in M9 medium and 0.7 h^{-1} in LB. At an OD_{540} of 0.5, expression was induced with 1 mM IPTG and aliquots of whole cells were sampled at 0, 1, 3 and 5 h after induction for SDS-PAGE and Western blot analysis (data not shown). A maximum level of expression of the hydroxylase component to about 15 % of the total cell protein was observed by SDS-PAGE after 3 h induction (data not shown). The expression levels were very similar to those observed in *E. coli* BL21(DE3) [pSMMO20] (Figure 4.2a) as judged by SDS-PAGE stained with Coomassie Brilliant Blue. Western blotting with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b confirmed the expression of the α, β and γ subunits of the hydroxylase as observed in *E. coli* BL21(DE3) [pSMMO20] (Figure 4.2b). The same result was observed with *P. putida* F1 [pSMMO20] grown in M9 medium.

Observation of cells by phase-contrast microscopy indicated that, during growth in the presence of Cm, their morphology was altered (Table 4.3). This caused problems when trying to determine the purity of *P. putida* F1 [pSMMO20]. Therefore, the morphology of both *P. putida* F1 and *P. putida* F1 [pSMMO20] were studied in batch culture during growth in different media and in the presence and

Table 4.3: Effect of Cm on the morphology of *P. putida* F1 [pSMMO20].

Growth media	Wild-type *P. putida* F1	*P. putida* F1 [pSMMO20]
LB	Highly motile rods	Highly motile rods
M9	Highly motile rods	Highly motile rods
LB + Cm^{500}	No growth	Non motile, short rods
M9 + Cm^{500}	No growth	Non-motile, short curved rods
LB + Toluene	Highly motile rods	Highly motile rods
LB + Cm^{500} + Toluene	No growth	Non-motile short rods

absence of Cm. Only in the presence of antibiotic was the change in morphology identified and so this was thought to be an effect of Cm. This antibiotic functions as an inhibitor of protein synthesis, by inhibiting peptidyl transferase, which catalyses the formation of peptide bonds during polypeptide chain elongation. To solubilise the antibiotic, ethanol was used, but this was found to have no effect on morphology when added to the growth medium. An additional control, to check that the non-motile, short rods were *P. putida*, was to grow the organisms on toluene, as described in Section 2.3.2. Since *P. putida* F1 possess TDO, it can utilise toluene as a sole carbon and energy source (Gibson *et al.*, 1968). In all cases *P. putida* F1 [pSMMO20] grown in the presence of Cm could also be cultured using toluene as a sole carbon and energy source. Propylene oxidation enzyme assays of *P. putida* F1 [pSMMO20] grown in M9 medium were performed to establish if expression of functional sMMO could be detected (Table 4.4). The same assays were performed with cells grown in LB and enzyme activities for recombinant protein B and the

Table 4.4: Complementation of soluble extracts of *P. putida* F1 [pSMMO20] with purified sMMO proteins from *Mc. capsulatus* (Bath).

Protein	Specific activity (nmol min^{-1} mg $protein^{-1}$)
A + B + C	144 ± 2.9
P. putida F1 soluble extract	0
Soluble extract F1 [pSMMO20]	0[a,b]
Soluble extract F1 [pSMMO20] + B + C	0[b]
Soluble extract F1 [pSMMO20] + A + C	5.5 ± 1.6
Soluble extract F1 [pSMMO20] + A + B	2.0 ± 0.3
Soluble extract F1 [pSMMO20] + A	1.5 ± 0.2
Soluble extract F1 [pSMMO20] + B	0[b]
Soluble extract F1 [pSMMO20] + C	0[b]
A + B	0
A + C	0
B + C	0

[a] No activity was detected after 90 min incubation. [b] Assays also performed at 30 °C with the same result. Purified proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase; Soluble extract F1 [pSMMO20], 10 mg of soluble extract of *P. putida* F1 [pSMMO20]. Assays were performed at 45 °C in 500 µl 25 mM MOPS, pH 7.0. 8 nmol of proteins A, B and C, 481 nmol of propylene and 224 µmol NADH were used. The GC retention time of propylene oxide was 1.94 min in all assays. The results are the mean of three experiments.

reductase were approximately the same. These results suggested that recombinant sMMO from *P. putida* F1 [pSMMO20] was inactive, due to the expression of inactive hydroxylase.

4.2.5 Expression of sMMO in other strains of *P. putida*

To establish the effect of host strain on the activity of recombinant sMMO, Jahng and Wood (1994) electroporated pSMMO20 into six different strains of *P. putida*. Of these six strains, only *P. putida* F1 [pSMMO20] resulted in TCE degradation. To continue this work, attempts were made to transform *P. putida* NCIMB 8859, *P. putida* NCIMB 10007 and *P. putida* NCIMB 8248 with pSMMO20. *P. putida* NCIMB 11767 was used as the positive transformation control in all cases. Varying the potential difference from 5 to 15 kV and the time constants from 5 to 25 ms in electroporation experiments using 0.1 cm cuvettes, only transformants of *P. putida* NCIMB 11767 were obtained. The reason for this was unclear, however, similar results have been obtained by Dr. T. Smith (University of Warwick, UK) who found that out of the four strains tested, only *P. putida* NCIMB 11767 could be transformed with plasmid DNA.

4.2.6 Expression of sMMO in *P. putida* F1 [pSMMO40]

To improve the stability of pSMMO20, Jahng *et al.* (1996) subcloned the *parB* killing locus (Gerdes, 1988) into this vector, generating pSMMO40. The *parB* locus encodes two gene products, *hok* and *sok* which encode a plasmid stabilisation system that can be used in many Gram negative organisms including *E. coli* and *P. putida*. The *parB* killing locus was effective since pSMMO40 was present in 61 % of cells, but pSMMO20 was present in only 9 % of cells after 12 h of cultivation. TCE degradation rates of *P. putida* F1 [pSMMO40] were 15-20 % higher than those observed for *P. putida* F1 [pSMMO20] (Jahng *et al.*, 1996). To establish if the increased stability of pSMMO40 could confer detectable propylene oxidation activity

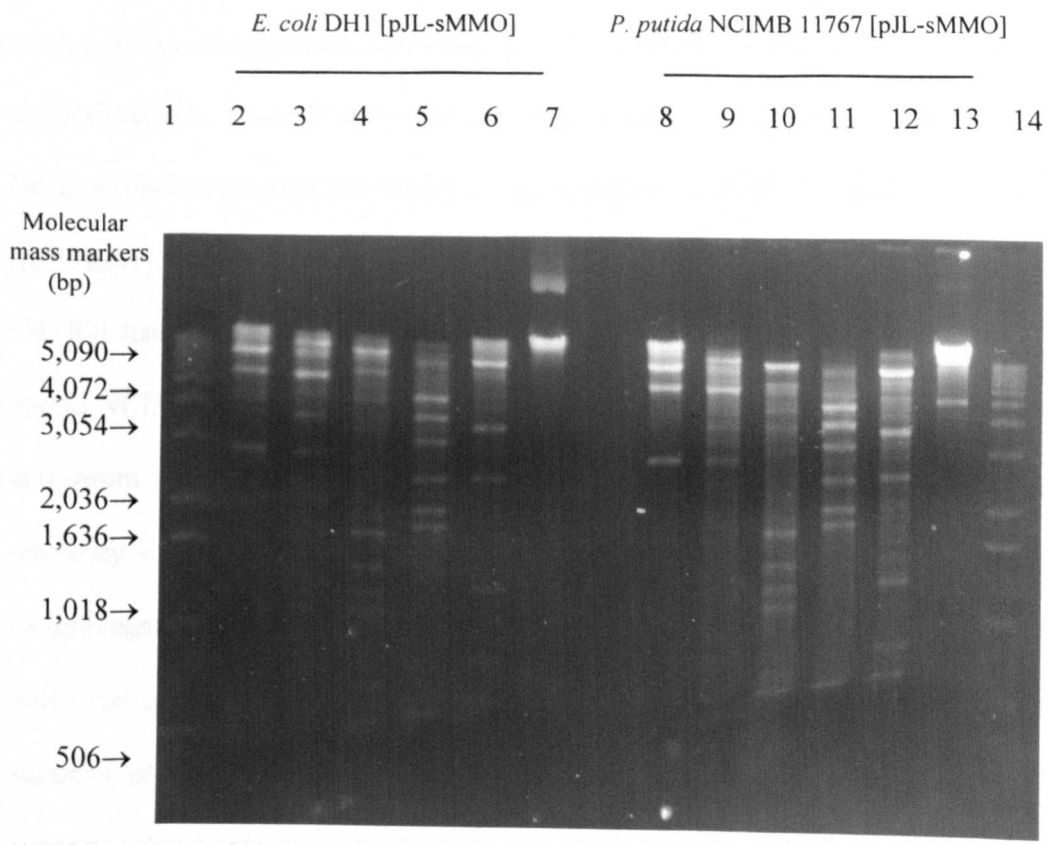
for recombinant sMMO, *E. coli* BL21(DE3) and *P. putida* NCIMB 11767 were transformed with pSMMO40. Transformants were grown in LB and soluble extracts were prepared after 5 h induction with 1 mM IPTG at 30 °C. Propylene oxidation assays were performed and results similar to those in Tables 4.1 and 4.2 were obtained. This suggested that the additional plasmid stability of pSMMO40 did not result in a detectable increase in enzyme activity for the recombinant hydroxylase, protein B or reductase.

4.3 Heterologous expression of sMMO from *Mc. capsulatus* (Bath) in *P. putida*

4.3.1 Expression of sMMO in *P. putida* NCIMB 11767 [pJL-sMMO]

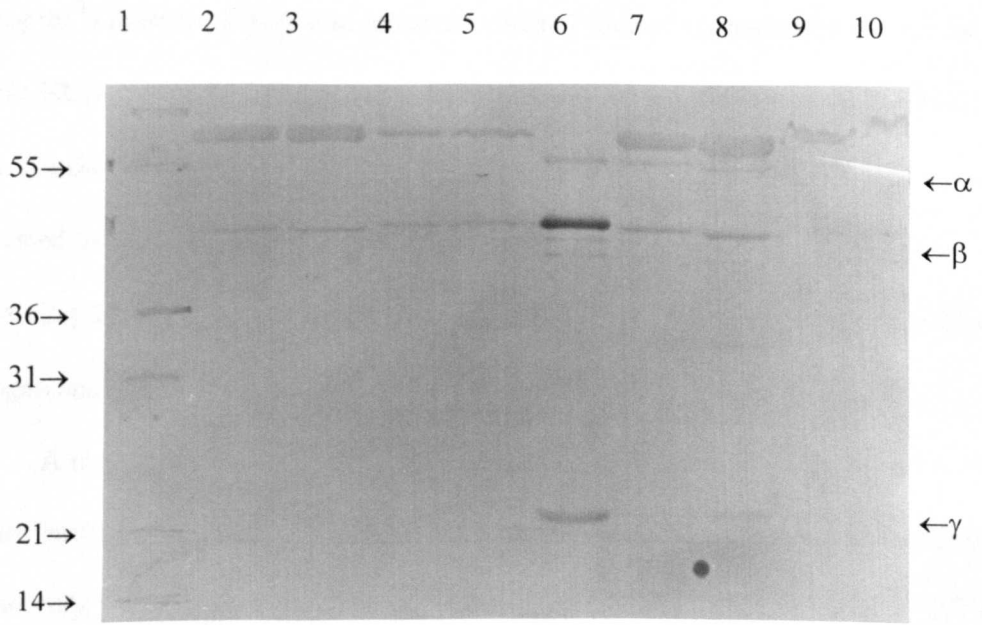
To determine if *P. putida* was a suitable heterologous host for the expression of functional sMMO from *Mc. capsulatus* (Bath), pJL-sMMO was constructed as described in Section 3.7. *E. coli* DH1 and *P. putida* NCIMB 11767 were electroporated with pJL-sMMO and transformants were selected on LB agar containing Ap (100 μg ml^{-1}) and Km (50 μg ml^{-1}). *E. coli* transformants appeared after overnight growth at 37 °C, compared to *P. putida* transformants which were obtained after 3 to 4 days incubation at 30 °C. No colonies were identified on antibiotic selection plates for wild-type *E. coli* DH1 and *P. putida* NCIMB 11767 controls. To ensure that the antibiotic resistance was conferred by pJL-sMMO, plasmid DNA was isolated using the method described in Section 2.7.1, and subsequently digested with restriction endonucleases (Figure 4.3). The restriction maps confirmed the integrity of the vector and that pJL-sMMO had been successfully transformed into both organisms. Expression of sMMO in *E. coli* DH1 [pJL-sMMO]

Figure 4.3: Restriction endonuclease digestion of pJL-sMMO from *E. coli* DH1 and *P. putida* NCIMB 11767. Lanes 1 and 14, 1 kb DNA ladder, lanes 2 to 7, pJL-sMMO from *E. coli* DH1 digested with *Bam*HI, *Eco*RI, *Pst*I, *Pvu*II, *Sal*I and *Sac*I respectively; lanes 8 to 13, pJL-sMMO from *P. putida* NCIMB 11767 digested with *Bam*HI, *Eco*RI, *Pst*I, *Pvu*II, *Sal*I and *Sac*I respectively.



is described in Section 3.7. *P. putida* NCIMB 11767 and *P. putida* NCIMB 11767 [pJL-sMMO] expressing recombinant sMMO from *Mc. capsulatus* (Bath), were grown in LB media at 30 °C and expression was induced at an OD_{540} of 0.5 with 1 mM IPTG. Whole cell lysates from samples of the culture taken at hourly intervals after IPTG induction were separated by SDS-PAGE and probed with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) (data not shown). Results confirmed that the antibody cross-reacted with the recombinant α, β and γ subunits of the hydroxylase, in whole cells lysates of *P. putida* NCIMB 11767 [pJL-sMMO] only. No cross-reactivity with the antibody was identified with *P. putida* NCIMB 11767 [pJB3Km1]. Copper-free growth (Section 2.4.2) of *P. putida* NCIMB 11767 [pJL-sMMO] had no effect on expression levels. Soluble and insoluble extracts of *P. putida* NCIMB 11767 [pJL-sMMO] were separated by SDS-PAGE and probed with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) (Figure 4.4). Cross-reactivity of the antibody was clearly seen with the α, β and γ subunits of the recombinant hydroxylase in soluble extracts of *P. putida* NCIMB 11767 [pJLsMMO] only. In contrast, *E. coli* DH1 [pJLsMMO] cross-reacted only with the β and γ subunits of the hydroxylase, in the soluble and insoluble extracts. This result suggested that *P. putida* NCIMB 11767 had the capacity to express all of the subunits of the *Mc. capsulatus* (Bath) hydroxylase, possibly in stoichiometric amounts and in a soluble form, since no cross-reactivity with the antibody was identified in the insoluble fraction. In addition, native-PAGE and Western blotting confirmed that the recombinant *Mc. capsulatus* (Bath) hydroxylase expressed in *P. putida* 11767 had the same electrophoretic mobility as the wild-type hydroxylase (data not shown).

Figure 4.4: Western blot of *Mc. capsulatus* (Bath) sMMO expression in *E. coli* DH1 and *P. putida* NCIMB 11767. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2 and 3, 15 and 30 μg of soluble extract of *E. coli* DH1 [pJL-sMMO]; lanes 4 and 5, 15 and 30 μg of insoluble extract of *E. coli* DH1 [pJL-sMMO]; lane 6, 2.5 μg of purified hydroxylase from *Mc. capsulatus* (Bath); lanes 7 and 8, 15 and 30 μg of soluble extract of *P. putida* NCIMB 11767 [pJL-sMMO]; lanes 9 and 10, 15 and 30 μg of insoluble extract of *P. putida* NCIMB 11767 [pJL-sMMO].



4.3.2 Purification of the recombinant sMMO hydroxylase of *Mc. capsulatus* (Bath) from *P. putida* NCIMB 11767 [pJL-sMMO]

Expression of *Mc. capsulatus* (Bath) sMMO genes in *P. putida* NCIMB 11767 [pJL-sMMO] could only be detected using Western blotting, confirming that the expression levels were very low (less than 1 % of the total cell protein). Using soluble extracts of *P. putida* NCIMB 11767 [pJL-sMMO] propylene oxidation assays at 45 °C were performed. No activity was detected for any of the sMMO components but this was attributed to the low level of expression of the recombinant sMMO. Therefore, attempts were made to purify the recombinant hydroxylase protein from *P. putida* NCIMB 11767 [pJL-sMMO] to establish if enzyme activity could be obtained. A 20 litre fermenter with 16 litre of LB containing Ap (100 μg ml^{-1}) and Km (50 μg ml^{-1}) (Section 2.4.2) was inoculated with 1 litre of an overnight culture of *P. putida* NCIMB 11767 [pJL-sMMO]. At an OD_{540} of 0.6, expression was induced with 1 mM IPTG and μ was 0.85 h^{-1}. After 5 h induction, cells were harvested and resuspended in a minimum volume of 25 mM MOPS, pH 7.0, containing 1 mM benzamidine and 5 mM DTT. Cells were drop frozen in liquid nitrogen and stored at -70 °C until required.

A major problem in attempting to purify the recombinant hydroxylase was that eluted fractions were unlikely to contain detectable propylene oxidation activity due to the very low expression levels of recombinant sMMO. This problem would be most apparent during the initial purification stages. Therefore, to monitor the purification steps SDS-PAGE and Western blotting with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) were used. To establish if the recombinant hydroxylase expressed in *P. putida* NCIMB 11767 [pJL-sMMO] could be purified in

the same way as the hydroxylase from wild-type *Mc. capsulatus* (Bath), DEAE and Mono Q ion exchange chromatography were initially used to test this hypothesis. sMMO had been routinely purified from *Mc. capsulatus* (Bath) using these media as described in Section 2.12.1. 88 g wet weight of cells were lysed *via* two passages through a cell disrupter (Section 2.13.1) and 4 g of soluble protein was obtained. The first DEAE purification step was essentially as described by Pilkington and Dalton (1990). Soluble extract was loaded onto a column of DEAE cellulose equilibrated with 25 mM MOPS pH 7.0, containing 1 mM benzamidine, 5 mM DTT and 50 mM NaCl. Material not binding to the column was eluted with the same buffer, yielding fractions that should contain the recombinant hydroxylase. SDS-PAGE and Western blotting with anti-serum against the hydroxylase from *Mc. capsulatus* (Bath) confirmed that this was the case (data not shown). Material from the DEAE column was then applied to a Mono Q column as described by Pilkington and Dalton (1990). The hydroxylase from *Mc. capsulatus* (Bath) eluted at approximately 150 mM NaCl (Pilkington and Dalton, 1990) and this was also the case for the recombinant hydroxylase from *P. putida* 11767 [pJL-sMMO]. Propylene oxidation activity was not detected.

In an attempt to improve the purification protocol, the remaining 2.5 g of soluble protein from *P. putida* 11767 [pJL-sMMO] was used with additional Q Sepharose high performance ion exchange and Superdex 200 gel filtration chromatography steps as described in Section 2.12.1 (Table 4.5). Results from the Superdex 200 chromatography step are shown in Figure 4.5. No propylene oxidation activity was detected but it was considered that this still could have been due to the low levels of recombinant hydroxylase present in the fraction. Fractions containing

Table 4.5: Partial purification of recombinant hydroxylase from *P. putida* NCIMB 11767 [pJL-sMMO].

Chromatography step	Elution of hydroxylase (mM NaCl)		Protein (mg)	Specific activity (nmol min^{-1} mg $protein^{-1}$)
	Mc. capsulatus (Bath)[1]	*P. putida* 11767 [pJL-sMMO]		
Soluble extract	NA	NA	2500	0
DEAE cellulose	50	50	820	ND
Q Sepharose	180-230	230-250	81	ND
Superdex 200	NA	NA	8	0
Mono Q	150	150	0.5	0

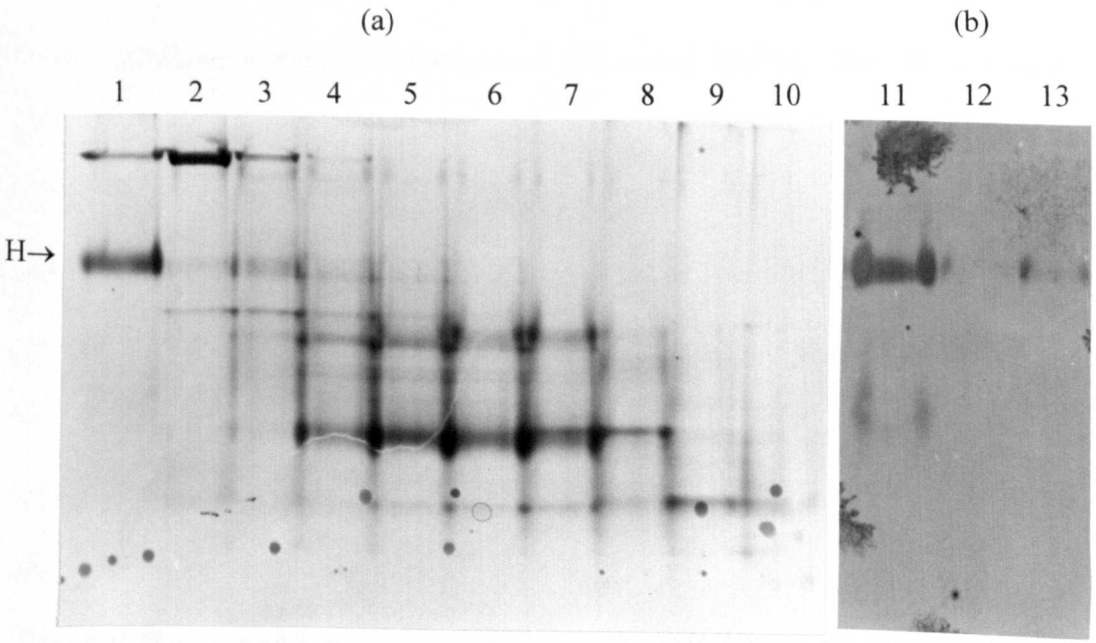
[1] Data obtained from Pilkington and Dalton (1990) and R. Titmus (University of Warwick, UK) for comparison. ND, not determined. NA. not applicable.

the hydroxylase were pooled, concentrated and the protein (8 mg) was applied to a Mono Q column. At 150 mM NaCl, approximately 0.5 mg of protein was eluted. This was used in propylene oxidation assays but no enzyme activity was detected.

4.4 Discussion

Heterologous expression of active sMMO from *Ms. trichosporium* OB3b in *P. putida* F1 [pSMMO20] was reported by Jahng and Wood (1994) and Jahng *et al.* (1996). This was determined using TCE, chloroform and propylene oxidation assays to detect recombinant sMMO enzyme activity. Such an expression system could have been ideal to use for site-directed mutagenesis studies of the hydroxylase component

Figure 4.5: (a) Native-PAGE and (b) Western blot of Superdex 200 partially purified recombinant sMMO hydroxylase from *P. putida* 11767 [pJL-sMMO], probed with anti-serum against the hydroxylase from *Ms. trichosporium* OB3b. Lane 1, 30 μg pure hydroxylase from *Ms. trichosporium* OB3b; lane 2, fraction 47; lane 3, fraction 48; lane 4, fraction 50; lane 5, fraction 52; lane 6, fraction 53; lane 7, fraction 56; lane 8, fraction 59; lane 9 fraction 62; lanes 11 to 13, Western blot of lanes 1, 2 and 3 respectively. H = Hydroxylase.



of sMMO.

The problem with using TCE degradation as an assay for recombinant sMMO activity in *P. putida* F1 (Jahng and Wood, 1994) was that the heterologous host was capable of TCE degradation. It is well established that toluene dioxygenase (TDO) of *P. putida* F1 degrades TCE (Nelson *et al.*, 1988; Wackett and Gibson, 1988; Zylstra *et al.*, 1989). The TDO genes are encoded by the *tod* operon (Zylstra *et al.*, 1988) and expression of these genes are only induced in the presence of toluene (Nelson *et al.*, 1987). However, recent studies have suggested that TCE can act as an inducer of toluene oxidising activity in *Pseudomonas* (Heald and Jenkins, 1994; McClay *et al.*, 1995; Leahy *et al.*, 1996). *P. putida* F1 grown in the absence of TCE had a specific toluene degradative activity of 7.1 ± 1.0 nmol min^{-1} mg $protein^{-1}$, in comparison to growth in the presence of 2.35 mM TCE, resulting in a specific toluene degradative activity of 41.1 ± 2.5 nmol min^{-1} mg $protein^{-1}$. This reflected 10.9 ± 1.6 % and 63 ± 3.9 % of the activity observed with cells grown in the presence of toluene, respectively (Leahy *et al.*, 1996). These results suggest that TCE or an oxidation product of TCE, induces TDO or other 'toluene degrading enzymes' in these strains. However, the work of Leahy *et al.* (1996) contrasted with the work of McClay *et al.* (1995) who found that TCE did not induce toluene-degradative activity in *P. putida* F1. It was proposed by Leahy *et al.* (1996) that this discrepancy may be due to differences in the growth of cells prior to assaying. Leahy *et al.* (1996) used resting cells that had been previously grown in the presence of 2.35 mM TCE whilst McClay *et al.* (1995) used cells grown in the absence of TCE which were then exposed to TCE as resting cells. The latter method was used by Jahng and Wood (1994).

To verify that TCE degradation was catalysed by sMMO, chloroform degradation was monitored since chloroform is oxidised by sMMO of *Ms. trichosporium* OB3b (Park *et al.*, 1991) but there are no reports of this substrate being metabolised by *P. putida* F1 expressing TDO. Chloroform degradation was observed with *P. putida* F1 [pSMMO20] induced with IPTG but not with *P. putida* F1 grown on toluene as a sole carbon and energy source. Controls of *P. putida* F1 grown on glucose or *P. putida* F1 [pSMMO20] not induced with IPTG were not included. The reason why the much simpler and reproducible propylene oxidation assay was not used at this stage by Jahng and Wood (1994) was unclear. However, it was concluded that the low activity of the recombinant sMMO may have been due to one or a combination of the following factors: (1) inefficient metal cofactor addition to the sMMO apoenzyme; (2) incorrect ratios of the sMMO components leading to incorrect aggregation; (3) conditions of cultivation such as lack of iron and cysteine (Jahng and Wood, 1994). Propylene oxidation assays of soluble extracts of *P. putida* F1 [pSMMO20] were published by Jahng *et al.* (1996). This confirmed that propylene oxidation activity was only detected after 90 min incubation and hence the specific activity of the recombinant sMMO was exceptionally poor (Table 4.6). Activity was detected when pure hydroxylase from *Ms. trichosporium* OB3b was added to soluble extracts of *P. putida* F1 [pSMMO20].

Results presented in this Chapter have confirmed that the majority of the recombinant hydroxylase protein was inactive. This was concluded because of the absence of activity using the propylene oxidation assay, which has been used in the majority of publications regarding the biochemistry and enzyme activity of sMMO. It was found that by adding pure hydroxylase from *Mc. capsulatus* (Bath) to soluble

Table 4.6: Complementation of soluble extracts of *P. putida* F1 [pSMMO20] with purified sMMO components from *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath).

Protein	Specific activity (nmol min^{-1} mg $hydroxylase^{-1}$) Jahng *et al.* (1996)[1]	Specific activity (nmol min^{-1} mg $hydroxylase^{-1}$) This study[2]
A + B + C	324	125
Soluble extract	0[3]	0[4]
Soluble extract + A	29	10
Soluble extract + B	0	0
Soluble extract + C	0	0
Soluble extract + A + B	42	13
Soluble extract + A + C	45	31

[1] Activity was measured after 18 min incubation at 30 °C using 10 nmol of pure A (hydroxylase), 20 nmol of pure B (protein B) and 10 nmol of pure C (reductase) from *Ms. trichosporium* OB3b.

[2] Activity was measured after 5 min incubation at 30 °C using 8 nmol of pure A, B and C from *Mc. capsulatus* (Bath), as described in Table 4.3.

[3] Propylene oxidation activity was identified after 90 min incubation.

[4] Propylene oxidation activity was not identified after 90 min incubation or at 30 or 45 °C.

extracts of *E. coli* and *P. putida* expressing recombinant sMMO from *Ms. trichosporium* OB3b, that enzyme activity was obtained and confirmed the results of Jahng *et al.* (1996). However, propylene oxidation activity was not detected even after 90 min incubation, in contrast to reports by Jahng *et al.* (1996) (Table 4.6).

Hence, recombinant protein B and recombinant reductase of *Ms. trichosporium* OB3b were functionally expressed in these heterologous hosts, but the hydroxylase was inactive. The same scenario for expression of sMMO from *Mc. capsulatus* (Bath) in *E. coli* (West *et al.*, 1992; Chapter 3) was therefore identified.

The expression of sMMO from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b has now been studied in *E. coli*. One of the major problems identified in the expression of sMMO from *Mc. capsulatus* (Bath) was the weak expression of the α subunit of the hydroxylase (Chapter 3). In direct contrast to this, expression of the α subunit of the hydroxylase component from *Ms. trichosporium* OB3b was seen in *E. coli*. Strong promoters i.e. T7 and *ptac* were used in both cases. The predicted Shine Dalgarno sequences for *mmoX* from both organisms are shown in Figure 4.6. The

Figure 4.6: Predicted Shine-Dalgarno sequences for *mmoX* of *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b.

UGUC**AGGAGG**AACAAGCAUGGCG *Ms. trichosporium* OB3b

UA**CGGAGG**AAACAAGTAAUGGCA *Mc. capsulatus* (Bath)

Predicted Shine-Dalgarno sequence in **bold**; initiation codon underlined. *E. coli* consensus is 5'...AGGAGG...3'.

Shine-Dalgarno sequence of *Ms. trichosporium* OB3b is identical to the consensus sequence of *E. coli* and it is located 7 bp from the AUG start codon, which is also the optimal distance in *E. coli*. In comparison to this, the Shine-Dalgarno sequence for

mmoX of *Mc. capsulatus* (Bath) differs in one position and is also located 9 bp from the AUG start codon. It could be postulated that the reason for poor expression of *mmoX* of *Mc. capsulatus* (Bath) in *E. coli* (in comparison to the higher expression level of *mmoX* from *Ms. trichosporium* OB3b) was due to poor recognition of the Shine Dalgarno sequence. This can be caused by the formation of interfering RNA secondary structures, however, sequences capable of forming such structures have not been identified 5' of *mmoX* (Stainthorpe *et al.*, 1989). In addition, good expression of the other *Mc. capsulatus* (Bath) sMMO genes was identified which have Shine-Dalgarno sequences with very poor homology to the *E. coli* consensus (Section 3.8). Another hypothesis could be that although the α subunit of the hydroxylase of *Ms trichosporium* OB3b was insoluble, it did not fold in a protease sensitive conformation and was not rapidly degraded by the cell.

Although the results of the expression of the sMMO operon of *Ms. trichosporium* OB3b in *P. putida* were disappointing, work was pursued with *P. putida* as a heterologous host for the expression of the sMMO operon from *Mc. capsulatus* (Bath). This was because *P. putida* possess enzymes that are structurally similar to sMMO, including toluene-2-monooxygenase (Newman and Wackett, 1995); phenol hydroxylase (Nordlund *et al.*, 1990b) and toluene-4-monooxygenase (Fox *et al.*, 1994). Based upon the homology of the amino acid sequences of these proteins, it has been predicted that the active sites of each of these enzymes contain dinuclear iron binding ligands, similar to the hydroxylase of sMMO. This would suggest that *P. putida* has the capacity to assemble these di-iron centres. Since *E. coli* expressed inactive recombinant hydroxylase, it was of interest to determine if this problem could be overcome by expressing recombinant sMMO in *P. putida*. It was clear that

expression of the sMMO operon from *Mc. capsulatus* (Bath) in *P. putida* NCIMB 11767 [pJL-sMMO] resulted in the expression of all three subunits of the hydroxylase in a soluble form. In addition, the apparent native molecular weight of the recombinant hydroxylase was similar to that of the pure hydroxylase from *Mc. capsulatus* (Bath). This suggested that the recombinant hydroxylase expressed in *P. putida* assembled in the native $\alpha_2\beta_2\gamma_2$ configuration, although the subunits of the hydroxylase could still have misfolded, giving rise to inactive protein. The major problem encountered was the low level of recombinant sMMO expression from the *lac* promoter. To overcome this problem, a 16 litre induction was performed to obtain enough soluble extract and so the recombinant hydroxylase could be purified. Propylene oxidation assays could then be used to determine if the protein was active. Elution of the recombinant hydroxylase during the purification was consistent with that obtained for the wild-type hydroxylase protein. Unfortunately, by the end of the purification there was only enough partially purified protein for a single assay and no sMMO activity was detected when the protein was reconstituted with pure protein B and pure reductase from *Mc. capsulatus* (Bath). A worthwhile future experiment would be to perform a 100 litre induction with *P. putida* NCIMB 11767 [pJL-sMMO] and to purify the recombinant hydroxylase from soluble extracts using the methods described in this Chapter. If no activity was detected during any of the purification stages then attempts to obtain activity could be made by reconstitution of the hydroxylase with iron (Atta *et al.*, 1993) for example (Section 3.5.4). Alternatively, broad host range RK2 expression vectors based upon pJB3Km1 have been recently constructed (Blatny *et al.*, 1997) that would be very useful in increasing the levels of recombinant protein expression in *P. putida*. In addition, co-expression of the sMMO

and GroESL operons (discussed in Section 3.8) from *Ms. trichosporium* OB3b in *E. coli* and *P. putida* could be performed to determine if the presence of this molecular chaperone would result in functional sMMO expression.

CHAPTER 5

Expression, purification and characterisation of recombinant sMMO proteins from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b

5.1 Introduction

Since the three components of sMMO can be purified separately and then reconstituted *in vitro* to give fully active sMMO, attempts were made to over-express and purify those sMMO proteins that were functionally expressed in *E. coli*, as fusion proteins. The expression of a protein fused to another protein, protein fragment or short polypeptide sequence has many advantages, including: (1) high level expression of the recombinant fusion protein; (2) rapid purification by affinity chromatography; (3) the fusion protein can be detected and quantified rapidly; (4) the recombinant protein often accumulates as a soluble protein rather than as inclusion bodies; (5) fusion proteins sometimes retain the biological activity of the native protein (Hockney, 1994). Many different vectors have been constructed for the expression of fusion proteins including glutathione S-transferase (GST) (Smith and Johnson, 1988), maltose binding protein (Nygren *et al.* 1994) and 6His (Hochuli *et al.* 1987).

It has been well established that protein B and the reductase of *Mc. capsulatus* (Bath) are functionally expressed in *E. coli* (West *et al.*, 1992; Chapter 3). Although sMMO from *Ms. trichosporium* OB3b was inactive in *E. coli*, protein B and the reductase were expressed in an active form in this organism (Jahng *et al.*, 1996; Chapter 4).

The aim of the work presented in this Chapter was to express protein B and the reductase from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b in *E. coli* as GST fusion proteins. This would facilitate their rapid purification and routine preparation from *E. coli*. In addition, the role of *orfY*, which lies between *mmoZ* and *mmoC* on the sMMO operon, is unknown. By over-expressing as a fusion protein and then

purifying recombinant OrfY, the protein could be characterised in an attempt to elucidate its role in methane oxidation.

5.2 PCR amplification and construction of vectors

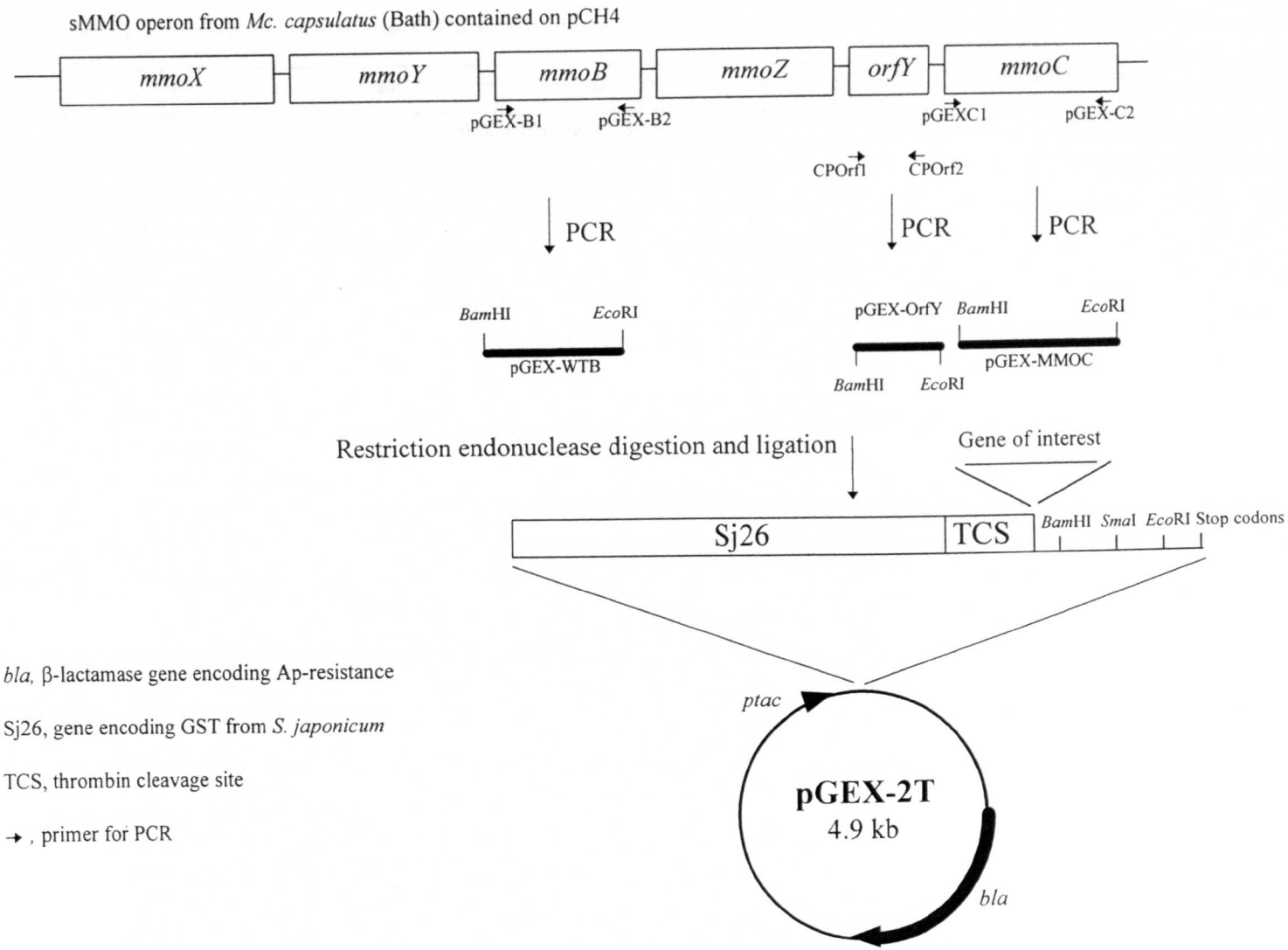
The vector used for the construction of GST fusion proteins was pGEX-2T which contains a 26 kDa GST gene (Sj26) of *Shistosoma japonicum* under the control of an IPTG inducible *tac* promoter (Pharmacia Biotech) (Figures 5.1 and 5.2). The normal termination codon is replaced by a polylinker followed by TGA translation termination codons in all three reading frames. PCR amplified genes were digested with the appropriate restriction enzyme(s) and then ligated in frame with the Sj26 gene of pGEX-2T that had been digested with the same restriction enzymes. Details of the PCR reactions and primers used are described in Table 5.1. After ligation and transformation into *E. coli* AD202 (Nakano *et al.*, 1994) colonies were screened by restriction analysis or colony hybridisation and the inserts were sequenced (using the forward primer, PGEX-958) to ensure the fidelity of the PCR. Transformants were screened for the expression of a GST fusion protein by analysing whole cell lysates after IPTG (1 mM) induction for 5 h at 37 °C.

5.3 Affinity purification of GST fusion proteins

5.3.1 Protein B from *Mc. capsulatus* (Bath)

Induction of pGEX-WTB at an OD_{540} of 0.5 with 1 mM IPTG at 37 °C had a deleterious effect upon the cell, causing cell death after 1 h induction possibly due to the toxicity of the protein to the cell (Figure 5.3). However, a protein with an approximate molecular mass of 43 kDa was expressed (Figure 5.4a). Western

Figure 5.1: Construction of *Mc. capsulatus* (Bath) GST-fusion vectors. Diagram not to scale.



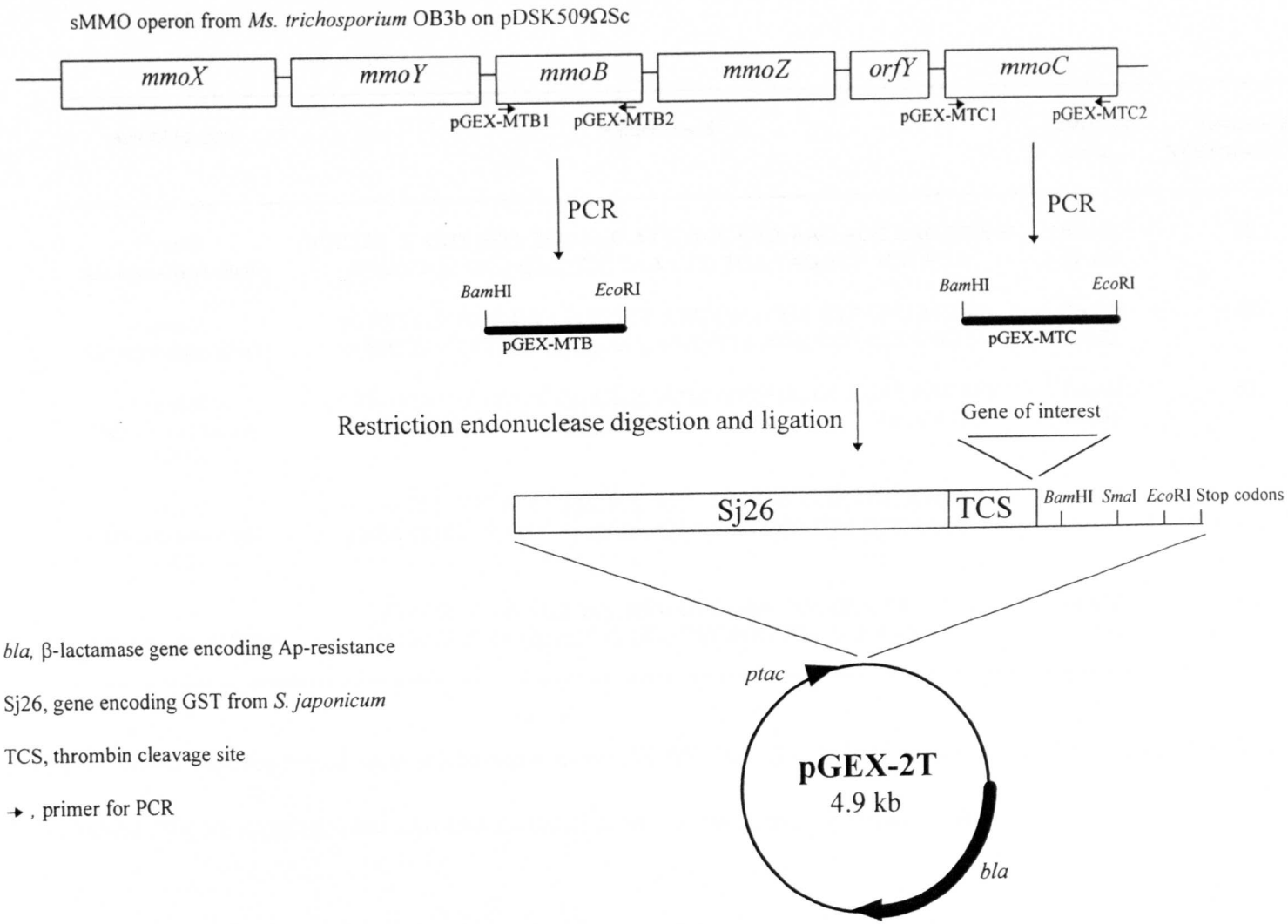
bla, β-lactamase gene encoding Ap-resistance

Sj26, gene encoding GST from *S. japonicum*

TCS, thrombin cleavage site

→ , primer for PCR

Figure 5.2: Construction of *Ms. trichosporium* OB3b GST-fusion vectors. Diagram not to scale.



bla, β-lactamase gene encoding Ap-resistance

Sj26, gene encoding GST from *S. japonicum*

TCS, thrombin cleavage site

→ , primer for PCR

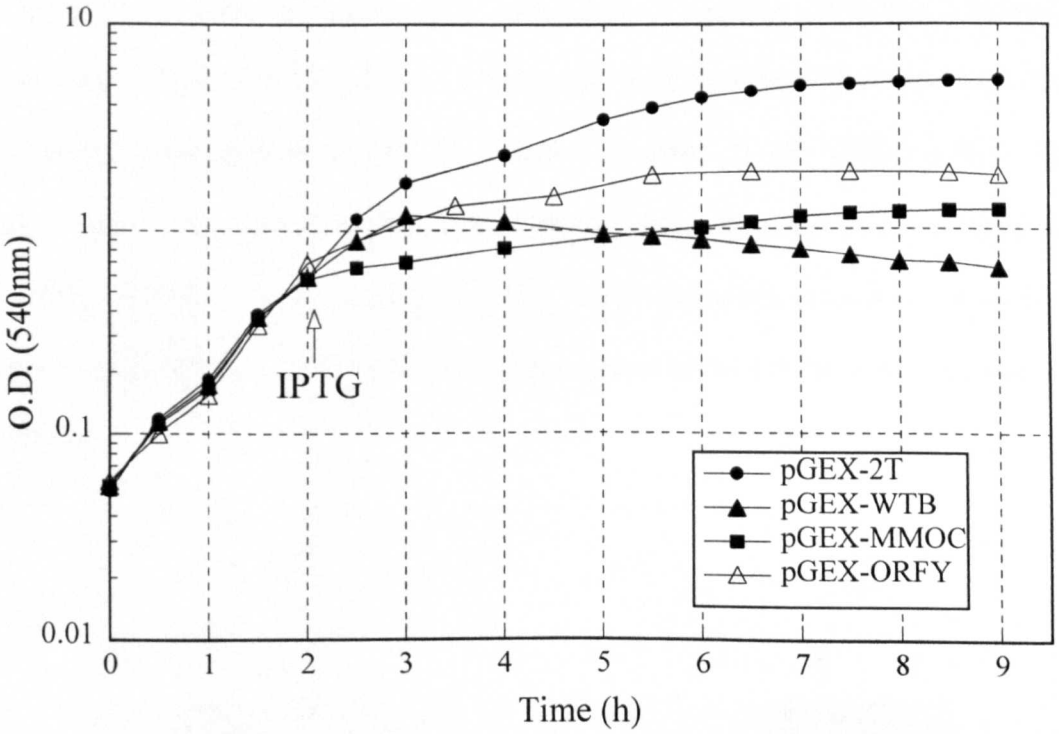
Table 5.1: PCR amplification of sMMO genes for the construction of GST fusion proteins.

Amplified gene	Primers used [1]	Restriction sites	Annealing temperature (°C)[2]	Vector name
mmoB *Mc. capsulatus* (Bath)	pGEXB1 5' GGT GGA TCC ACG **ATG** AGC GTA AAC AGC AAC GCA 3' pGEXB2 5' GGC GAA TTC TAA GCG TGA TAG TCT TCG AG 3'	*Bam*HI *Eco*RI	55	pGEX-WTB
mmoC *Mc. capsulatus* (Bath)	pGEXC1 5' GCC GGA TCC TTT **ATG** CAG CGA GTT CAC ACT 3' pGEXC2 5' CCG GAA TTC CCC CGG TTC AGG CCG CCC CGG 3'	*Bam*HI *Eco*RI	62	pGEX-MMOC
mmoB *Ms. trichosporium* OB3b	pGEX-MTB1 5' G ATC GGA TCC **ATG** TCC AGC GCT CAT AAC G 3' pGEX-MTB2 5' GAT CGA ATT CCG ATC AGA TGT CGG TCA G 3'	*Bam*HI *Eco*RI	55	pGEX-MTB
mmoC *Ms. trichosporium* OB3b	pGEX-MTC1 5' G ATC GGA TCC **ATG** TAC CAG ATC GTC ATC GA 3' pGEX-MTC2 5' GAT ACC CGG GAT CAG CCG CTC GCC AGG 3'	*Bam*HI *Sma*I	55	pGEX-MTC
orfY *Mc. capsulatus* (Bath)	CPOrf1 5' TG GGA TCC **ATG** GTC GAA TCG GCA TTT 3' CPOrf2 5' GG GAA TTC GCG CTT ACC TGT TCA ATG 3'	*Bam*HI *Eco*RI	40	pGEX-OrfY

pGEX-2T inserts were sequenced using the forward primer, PGEX-958: 5' CCA TCC TCC AAA ATC GGA TCT GGT 3'

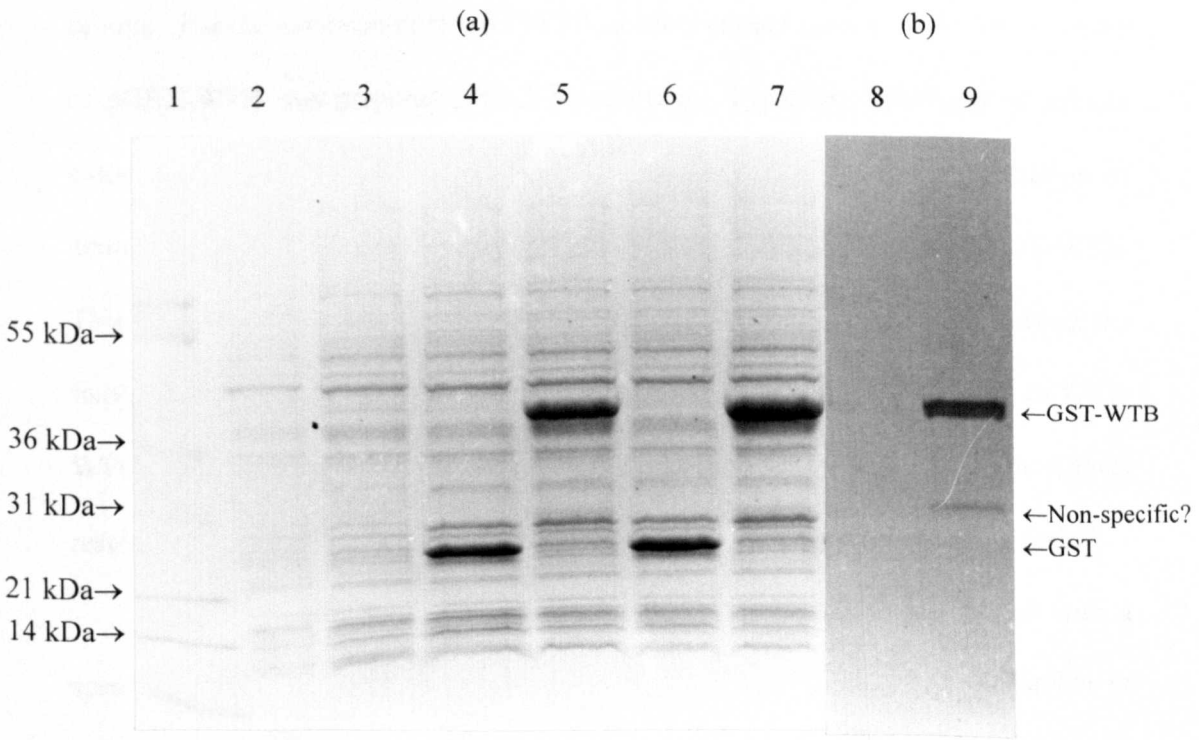
[1] Restriction sites are underlined and start codons are in **bold**; [2] Experimentally determined.

Figure 5.3: Growth curves of *E. coli* AD202 expressing GST fusion proteins from *Mc. capsulatus* (Bath).



blotting of whole cell lysates with anti-serum against protein B from *Mc. capsulatus* (Bath) confirmed that this protein cross-reacted with the antibody and probably represented the GST fusion protein (GST-WTB) (Figure 5.4b). To confirm that the molecular mass of GST-WTB was correct, ESI-MS was used. A molecular mass was first obtained for GST, the expected mass being 26,854.87 Da but the observed mass was 27,084.63 ± 3.51 Da. This increase in molecular mass of 229.8 Da could not be assigned to a specific post-translational modification. The molecular mass of GST-WTB determined by ESI-MS was 43,292 Da in comparison to an expected molecular mass of 42,246.05 Da. Due to the difference between the expected and observed molecular masses, an exact molecular mass for GST-WTB could not be assigned. A

Figure 5.4: (a) SDS-PAGE and (b) Western blot of *Mc. capsulatus* (Bath) protein B fusion protein expressed in *E. coli* AD202. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2, 4 and 6, *E. coli* AD202 [pGEX-2T], 0, 1, and 3 h respectively after IPTG induction, lanes 3, 5 and 7, *E. coli* AD202 [pGEX-WTB], 0, 1 and 3 h respectively after IPTG induction; lanes 8 and 9, lanes 6 and 7 respectively, probed with anti-serum against protein B from *Mc. capsulatus* (Bath).

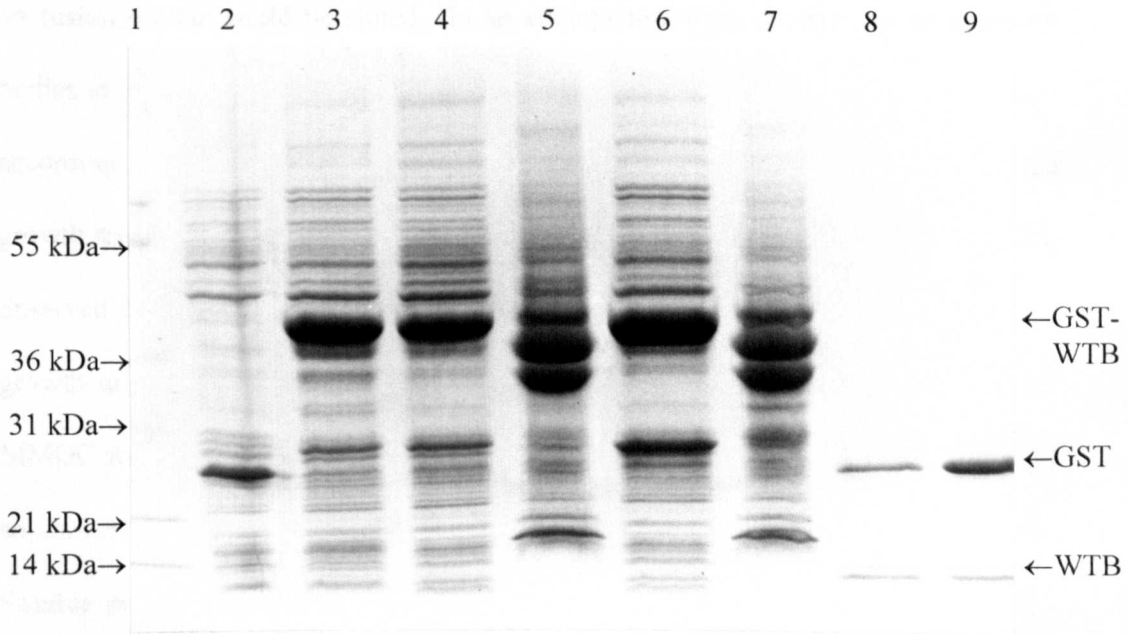


molecular mass of 16,227.03 ± 7.22 Da was determined for WTB (cleaved from GST-WTB with thrombin as described in Section 2.10.2) compared to an expected molecular mass of 16,229.02 Da.

During induction, the formation of inclusion bodies was monitored and after 5 h induction about 10 % of the culture contained at least one inclusion body per cell. To determine if an appreciable amount of GST-WTB was accumulating in the cell as insoluble protein, soluble and insoluble extracts of induced cells were prepared. These were separated by SDS-PAGE (Figure 5.5) and it was concluded that greater than 90 % of GST-WTB was present in the soluble fraction. It was therefore considered unnecessary to optimise the growth conditions for expression of soluble protein. For the purification of GST-WTB, soluble extract from a 1 litre batch culture of pGEX-WTB was prepared after 3 h induction. Typically, 85-95 mg of soluble extract (the mean of five experiments) was obtained at a protein concentration of about 25 mg ml^{-1}. A typical purification yielded 10-12 mg of purified GST-WTB. This was comparable to the yields reported by Smith and Johnson (1988) using the same affinity chromatography system. Propylene oxidation enzyme assays of GST-WTB, reconstituted with the hydroxylase and reductase from *Mc. capsulatus* (Bath) resulted in a specific activity for GST-WTB of 5,700 ± 340 nmol min^{-1} mg $protein^{-1}$.

From a 1 litre batch culture, 3-6 mg of purified WTB was obtained with a specific activity of 8,800 ± 410 nmol min^{-1} mg $protein^{-1}$. This was comparable to protein B from *Mc. capsulatus* (Bath) which has a specific activity of 7,289 nmol min^{-1} mg $protein^{-1}$ (Green and Dalton, 1985). Further characterisation of recombinant protein B from *Mc. capsulatus* (Bath) is described in Section 6.5.

Figure 5.5: SDS-PAGE of soluble and insoluble extracts of *E. coli* AD202 [pGEX-WTB]. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2, whole cell lysate of *E. coli* AD202 [pGEX-2T] after 3 h induction; lanes 3, 4 and 6, 20, 40 and 60 μg respectively of *E. coli* AD202 [pGEX-WTB] soluble extract after 3 h induction; lanes 5 and 7, 20 and 40 μg respectively of *E. coli* AD202 [pGEX-WTB] insoluble extract after 3 h induction; lanes 8 and 9, 15 and 30 μg of pGEX-WTB, cleaved with 30 ng thrombin for 10 min at 20 °C.



5.3.2 Reductase from *Mc. capsulatus* (Bath)

E. coli AD202 [pGEX-MMOC] was induced with 1 mM IPTG at 37 °C and aliquots were analysed by SDS-PAGE (data not shown). A protein with a molecular mass of about 65 kDa was expressed which was approximately the predicted molecular mass for the GST-MMOC fusion protein. During IPTG induction, the formation of inclusion bodies was monitored and after 5 h approximately 90 % of the culture contained refractile bodies. SDS-PAGE of soluble and insoluble extracts indicated that all of the detectable GST-MMOC fusion protein was in the insoluble fraction (data not shown). To determine if any soluble GST-MMOC could be purified, affinity chromatography with Glutathione Sepharose 4B was used, however, no fusion protein could be eluted. In an attempt to reduce the number of inclusion bodies in the culture and obtain soluble protein, the growth conditions were optimised according to the method of Schein and Noteborn (1988) (Table 5.2). Reducing the growth temperature and IPTG concentration reduced the number of inclusion bodies observed in the culture. SDS-PAGE of soluble and insoluble extracts prepared after growth under the different conditions described in Table 5.2 confirmed that GST-MMOC was only identified in the insoluble extract (data not shown). In an attempt to solubilise the fusion protein, the method described in Section 2.11.5 was used. Soluble protein was eluted from Q Sepharose in 100 mM increments from 250 to 750 mM NaCl. GST-MMOC was not identified in the soluble fractions after the attempted solubilisation (data not shown). In addition, the GST fusion protein could not be purified from the soluble extracts using affinity chromatography (Section 2.11.2). Although it may have been possible to solubilise and renature GST-MMOC from inclusion bodies, this method was not attempted. Instead, recombinant *Mc.*

Table 5.2: Effect of growth conditions upon inclusion body formation of GST-MMOC in *E. coli* AD202.

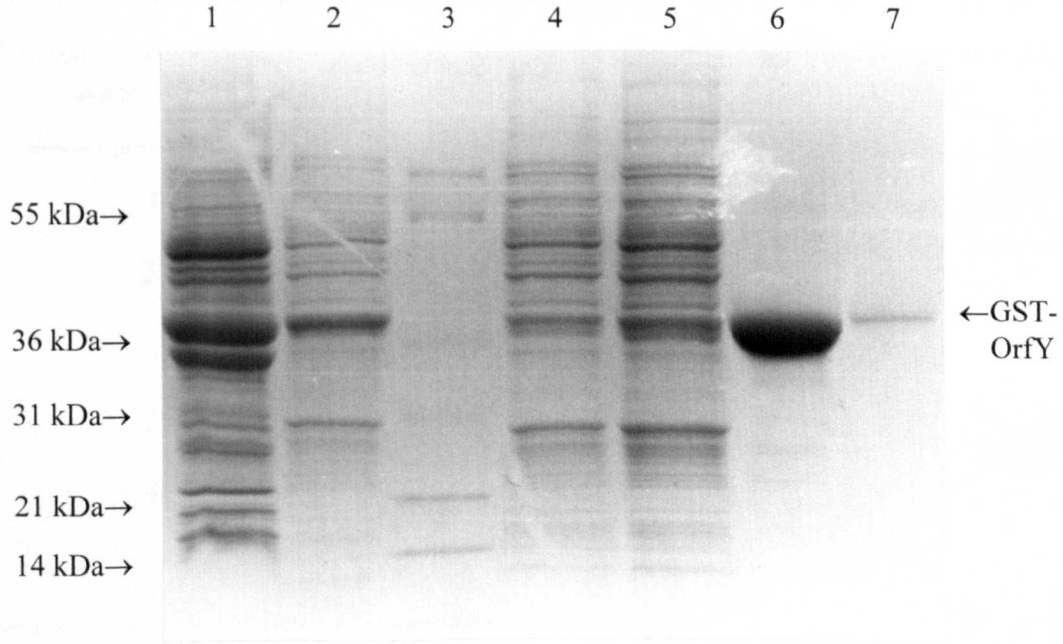
Time (h)	Number of cells containing inclusion bodies (%)			
	25 °C 0.1 mM IPTG	25 °C 1 mM IPTG	30 °C 0.1 mM IPTG	30°C 1 mM IPTG
0	0	0	1	5
1	0	0	3	10
2	2	0	5	20
3	5	1	5	30
4	5	5	10	30
5	10	10	20	40

capsulatus (Bath) reductase was routinely obtained using *E. coli* BL21(DE3) [pEC71] (West *et al.*, 1992).

5.3.3 OrfY from *Mc. capsulatus* (Bath)

E. coli AD202 [pGEX-OrfY] was induced with 1 mM IPTG at an OD_{540} of 0.5 for 5 h. Aliquots were taken during the time course and analysed by SDS-PAGE which showed that a fusion protein with a molecular mass of approximately 38 kDa was expressed. From a 1 litre batch culture, 4-6 mg of purified GST-OrfY was obtained. Analysis of the soluble and insoluble extracts (Figure 5.6) indicated that greater than 80 % of GST-OrfY was present in the soluble fraction which was consistent with a low level of inclusion body formation (approximately 20 % of cells

Figure 5.6: Purification of GST-OrfY from *E. coli* AD202 [pGEX-OrfY]. Lane 1, 25 μg of *E. coli* AD202 [pGEX-OrfY] insoluble extract; lane 2, 25 μg of *E. coli* AD202 [pGEX-OrfY] soluble extract; lane 3, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 4, 25 μg protein from first column wash; lane 5, 25 μg protein from second column wash; lane 6, 25 μg first elution of GST-OrfY; lane 7, 10 μg second elution of GST-OrfY.



contained inclusion bodies after 5 h induction). Soluble GST-OrfY was purified as detailed in Figure 5.7. From a 1 litre batch culture, 2-5 mg of purified OrfY was obtained. In an attempt to elucidate whether OrfY had any effect on sMMO activity, recombinant GST-OrfY and OrfY were added to pure sMMO proteins from *Mc. capsulatus* (Bath) and propylene oxidation assays were performed (Tables 5.3 and 5.4). The data obtained implied that both GST and GST-OrfY caused inhibition of

Table 5.3: Effect of GST and GST-OrfY upon sMMO propylene oxidation activity.

Amount of GST/GST-OrfY added (nmol)	Activity of A + B + C + GST-OrfY (%)	Activity of A + B + C + GST (%)
0	100	100
2.5	74	89
5	64	88
7.5	28	71
10	23	66
12.5	20	57

Pure proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase. Assays were performed at 45°C in 500 µl 25 mM MOPS, pH 7.5 with 6 nmol each of A, B and C, 481 nmol of propylene and 224 µmol NADH. 100 % activity represents 109 ± 15 nmol propylene oxide min^{-1}. The results are the mean of three assays. Standard error was ± 5 %.

Table 5.4: Effect of OrfY and bovine serum albumin (BSA) upon sMMO propylene oxidation activity.

Amount of OrfY/BSA added (nmol)	Activity of A + B + C + OrfY (%)	Activity of A + B + C + BSA (%)
0	100	100
2.5	73	86
5	56	72
7.5	55	73
10	48	67
12.5	49	64

Pure proteins from *Mc. capsulatus* (Bath): A, hydroxylase; B, protein B; C, reductase. Assays were performed at 45°C in 500 µl 25 mM MOPS, pH 7.5 with 6 nmol each of A, B and C, 481 nmol of propylene and 224 µmol NADH. 100 % activity represents 115 ± 9 nmol propylene oxide min^{-1}. The results are the mean of three assays. Standard error was ± 7 %.

sMMO activity, but GST-OrfY caused a two-fold reduction in sMMO activity when compared to GST alone. To elucidate if the same effect was seen with recombinant OrfY, the same experiment was repeated, using BSA as the control protein (Table 5.4). BSA and OrfY inhibited the enzyme activity of sMMO. It was therefore concluded from these data that OrfY had a negligible effect upon the activity of the enzyme. Other enzyme assays were performed to establish if OrfY could replace the role of either the hydroxylase, protein B or the reductase. Results indicated that recombinant OrfY could not replace the role of any of these proteins.

5.3.3.1 Anti-serum preparation for recombinant OrfY from *Mc. capsulatus* (Bath)

To elucidate whether *orfY* was transcribed and translated in *Mc. capsulatus* (Bath), antibodies were prepared for use in Western blotting experiments. Uncleaved GST-OrfY was used to raise antibodies due to the potential toxic effect of the serine protease inhibitor, thrombin. The cross-reactivity of anti-serum against GST-OrfY with recombinant OrfY protein is shown in Figure 5.7. When *orfY* was over-expressed from a T7 promoter using pT7-5OrfY, accumulation of OrfY to about 5 % of the total cell protein was identified after 5 h induction with 1 mM IPTG at 37 °C (data not shown). Although non-specific cross-reactivity was observed with other *E. coli* proteins, cross-reactivity between recombinant OrfY and anti-serum against GST-OrfY was identified (data not shown). The polyclonal anti-serum cross-reacted non-specifically with other *E. coli* proteins because the original injection of GST-OrfY was purified by affinity chromatography and was only 70-80 % pure as judged by SDS-PAGE stained with Coomassie Brilliant Blue. Anti-sera with a higher specificity against GST-OrfY could have been obtained if the protein had been purified further by Mono Q ion exchange chromatography for example, or by using immunoaffinity purification of the antibodies on an antigen column (Harlow and Lane, 1988). No cross-reactivity was observed using pre-immune serum.

To establish whether OrfY was expressed in *E. coli* during expression of the whole sMMO operon, a time course of induction of *E. coli* BL21(DE3) [pT7-5sMMO] was performed. Whole cell lysates were separated by SDS-PAGE and Western blotted with 50 µl of anti-serum against GST-OrfY (Figure 5.8). Although non-specific cross-reactivity was identified, weak cross-reactivity with a protein of

Figure 5.7: SDS PAGE and Western blotting of pure OrfY from *E. coli* AD202. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, 25 μg *E. coli* AD202 [pGEX-2T] soluble extract; lane 3, 25 μg *E. coli* AD202 soluble extract; lane 4, 20 μg purified OrfY; lanes 5 to 6, Western blot of lanes 3 and 4 probed with 50 μl of anti-serum against GST-OrfY.

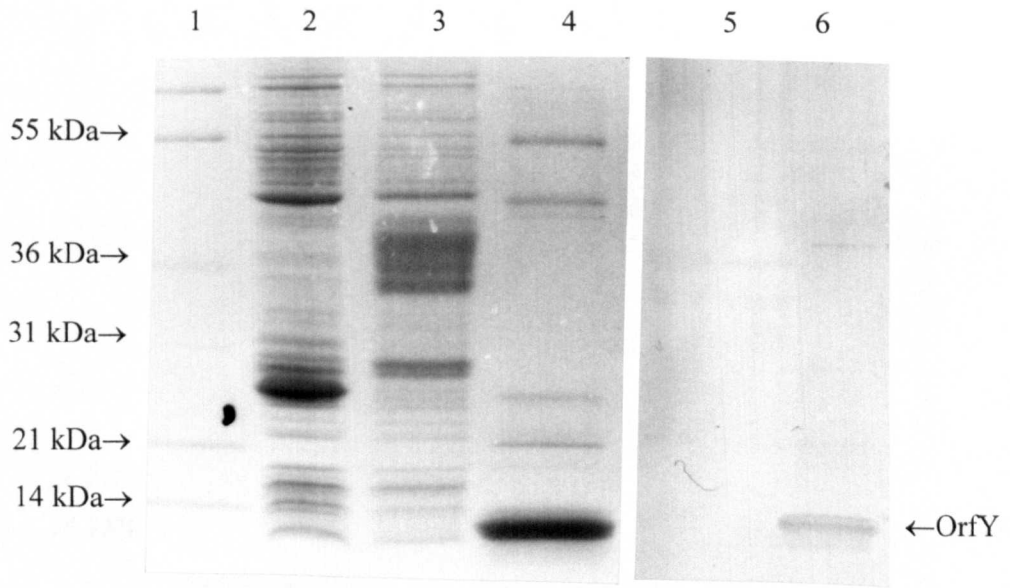
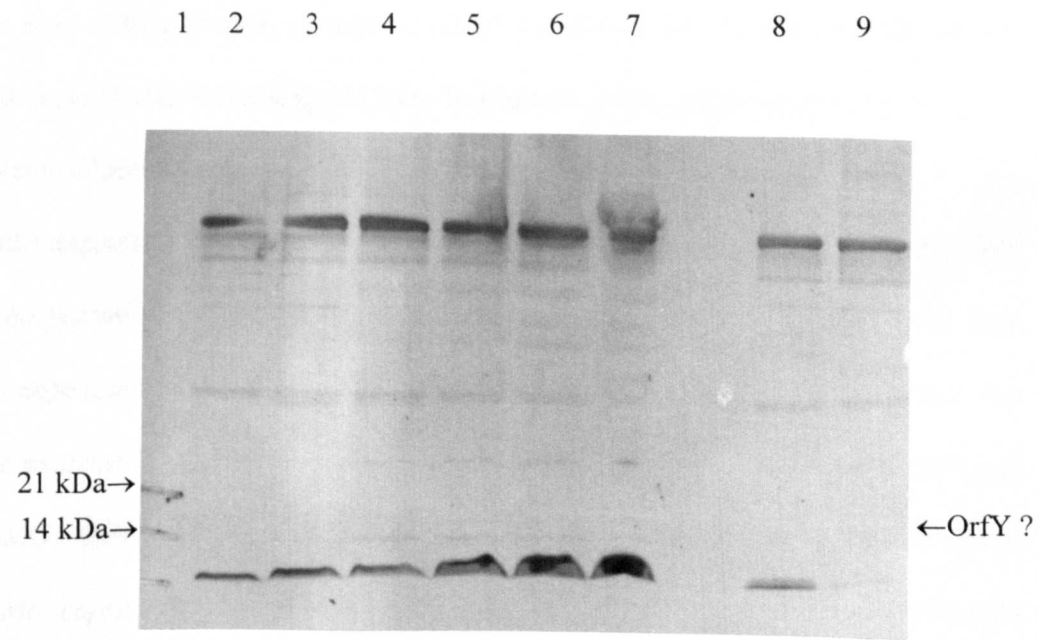


Figure 5.8: Western blot of *E. coli* BL21(DE3) [pT7-5sMMO] probed with anti-serum against GST-OrfY. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2 to 7, 40 μg *E. coli* BL21(DE3) [pT7-5sMMO] 0, 1, 2, 3, 4 and 5 h respectively after induction with IPTG; lanes 8 and 9, 40 and 20 μg of *E. coli* BL21(DE3).



about 14 kDa occurred in *E. coli* BL21(DE3) [pT7-5sMMO] and not with the control *E. coli* BL21(DE3). This provided tentative evidence that *orfY* was transcribed and translated in *E. coli* BL21(DE3) [pT7-5sMMO].

To establish whether OrfY was expressed in methanotrophs, Western blots were performed with anti-serum against the GST-OrfY protein and the soluble and insoluble extracts of *Mc. capsulatus* (Bath) expressing sMMO and pMMO respectively (data not shown). In addition, soluble extract from sMMO expressing *Ms. trichosporium* OB3b was used. Cross-reactivity of anti-serum against GST-OrfY was seen with a protein of approximately 42 kDa in the insoluble extract of *Mc. capsulatus* (Bath) expressing pMMO. In addition, cross-reactivity was seen with two proteins of approximately 42 kDa and 50 kDa in the soluble extract of *Mc. capsulatus* (Bath) expressing sMMO. No cross-reactivity was seen with soluble extracts of *Ms. trichosporium* OB3b expressing sMMO (data not shown). The expected result from this experiment would have been the identification of a protein of about 14 kDa that was expressed only under conditions when *Mc. capsulatus* (Bath) was expressing sMMO. This would have been consistent with the hypothesis that the sMMO operon of *Mc. capsulatus* (Bath) was transcribed from a single copper regulated promoter upstream of *mmoX* (Nielsen *et al.*, 1996, 1997). The results obtained could have implied that OrfY was subject to a post-translational modification, since the 14 kDa monomer was not identified. To determine if OrfY was associated with an sMMO specific protein, a Western blot was performed with pure sMMO proteins in addition to soluble extract from sMMO expressing *Mc. capsulatus* (Bath) that had been separated into three active fractions by DEAE cellulose chromatography (provided by

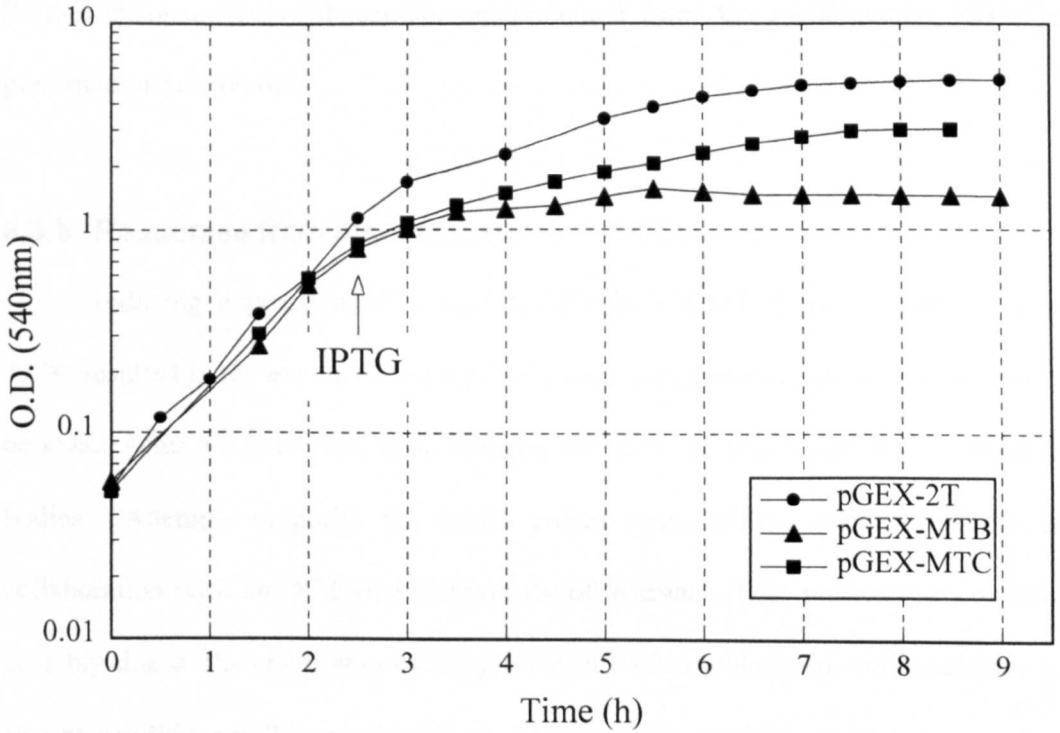
R. Titmus, University of Warwick, UK) (data not shown). Cross-reactivity was seen in all cases with DEAE fractions but not pure sMMO proteins (data not shown).

The work performed with recombinant OrfY and GST-OrfY antibody did not succeed in elucidating a role for *orfY*. Although results suggested that OrfY was expressed in *E. coli* BL21(DE3) [pT7-5sMMO], there was no evidence that suggested this was the case for *Mc. capsulatus* (Bath). However, further work is currently in progress (S. Harris and J. C. Murrell, personal communication) to establish if *orfY* is transcribed and translated in *Mc. capsulatus* (Bath) and will be discussed in Section 5.4.

5.3.4 Protein B from *Ms. trichosporium* OB3b

Induction of pGEX-MTB (the plasmid encoding the *Ms. trichosporium* OB3b protein B-GST fusion), at an OD_{540} of 0.5 with 1 mM IPTG at 30 °C resulted in the expression of a fusion protein with a molecular mass of about 40 kDa as judged by SDS-PAGE. Over-expression of *mmoB* from *Ms. trichosporium* OB3b did not have a detrimental affect upon cell growth (in comparison to *mmoB* from *Mc. capsulatus* (Bath) (Section 5.3.1) (Figure 5.9)). It was not possible to use Western blotting to determine if the fusion protein contained protein B from *Ms. trichosporium* OB3b since no antibody was available and the anti-serum against protein B from *Mc. capsulatus* (Bath) showed no cross-reactivity. To ensure that the correct protein was being over-expressed in *E. coli*, ESI-MS was used to confirm the molecular mass. Due to the problems encountered with GST (Section 5.4), ESI-MS was not performed on GST-MTB, however, a molecular mass for MTB of 15,026.5 ± 2.4 Da was obtained, in comparison to an expected molecular mass of 15,027.08 Da. This

Figure 5.9: Growth curves of GST fusion proteins from *Ms. trichosporium* OB3b expressed in *E. coli* AD202.



confirmed that MTB was of the correct molecular mass and no errors were introduced during PCR. The latter was confirmed using DNA sequencing by Dr. S. Harris (University of Warwick, UK).

After 5 h induction, approximately 30 % of the culture contained inclusion bodies, suggesting that a proportion of GST-MTB was being expressed as insoluble protein. From a 1 litre batch culture, about 100 mg of soluble extract was obtained, from which approximately 8-10 mg of GST-MTB was purified. Propylene oxidation enzyme assays of GST-MTB performed at 45 °C, reconstituted with pure hydroxylase and pure reductase from *Mc. capsulatus* (Bath) resulted in a negligible amount of enzyme activity. From a 1 litre batch culture, 4-6 mg of MTB was obtained, which

had a specific activity of 6,400 nmol min^{-1} mg $protein^{-1}$ when assayed at 45 °C, with 8 nmol pure hydroxylase and 8 nmol pure reductase from *Mc. capsulatus* (Bath). Further characterisation of recombinant protein B from *Ms. trichosporium* OB3b is presented in Section 6.9.

5.3.5 Reductase from *Ms. trichosporium* OB3b

Inducing expression of *E. coli* AD202 [pGEX-MTC] with 1 mM IPTG at 37 °C resulted in the expression of a protein with a molecular mass of approximately 66 kDa. After 5 h induction, approximately 80 % of the culture contained inclusion bodies. Attempts to purify the fusion protein using affinity chromatography in collaboration with Dr. S. Harris (University of Warwick, UK) proved unsuccessful, possibly due to the expression of the protein in an insoluble form. Manipulation of growth conditions will be required in an attempt to obtain soluble protein.

5.4 Discussion

The use of fusions with GST proved to be a rapid and simple method for the heterologous expression and purification of recombinant sMMO proteins. However, the technique was limited by the fact that translational fusions can only be constructed between GST and a single open reading frame, due to the stop codon at the 3' end of the cloned gene. It was also limited by the inability of *E. coli* to express some recombinant proteins in a soluble form.

Purification of the *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b reductases were problematic owing to the expression of insoluble protein in *E. coli* which could have occurred because the reductases contain FAD and Fe_2S_2 (Colby and

Dalton, 1979). A common problem with expressing heterologous proteins in *E. coli* is that *E. coli* often lacks the capacity to assemble prosthetic groups correctly (Weickert, 1996). The problem of insoluble reductase from *Mc. capsulatus* (Bath) was also identified when *mmoC* was over-expressed in *E. coli* using a T7 promoter (West *et al.*, 1992), although some soluble protein could be purified. In an attempt to solubilise the GST-MMOC protein, a lower temperature of induction (Schein and Noteborn, 1988) and solubilisation of proteins using ion exchange resin (Hoess *et al.*, 1988) were used without success. A strategy that could have been used was the solubilisation of inclusion bodies with urea, guanidine hydrochloride, or SDS followed by renaturation and affinity purification (Hartman *et al.*, 1992). However, the recombinant reductase from *Mc. capsulatus* (Bath) could be purified from *E. coli* BL21(DE3) [pEC71] (West *et al.*, 1992), if solubilisation of GST-MMOC proves impossible in the future. Alternatively, a fusion could be constructed with an alternative protein such as thioredoxin for example, which has been shown (in some circumstances) to circumvent inclusion body formation in *E. coli* (LaVallie *et al.*, 1993). Since thioredoxin is a highly soluble and stable protein, it has been suggested that it may interact in some way with the fused protein, perhaps by acting as a covalently linked chaperone protein. Other techniques that could be used to improve solubility may include co-expression of thioredoxin and GroE, encoded by pT-Trx and pT-GroE, respectively (as described in Sections 3.5.3 and 3.5.5).

Of the three proteins that were functionally expressed in *E. coli*, one retained enzyme activity even when fused to GST. One may expect that the GST fusion protein would be translated in such a way that the conformation of the fused protein would be disrupted, rendering the protein inactive. Clearly, evidence suggests that

this is not the case and that both proteins can fold correctly even when fused together. This may suggest that the folding of GST is very resistant to any perturbations that may be caused by a fused protein. In the case of protein B, the GST-MMOB fusion protein was active when reconstituted with pure hydroxylase and pure reductase from *Mc. capsulatus* (Bath). In contrast, GST-MTB was inactive when reconstituted in the same manner as for GST-MMOB and was only active when the GST fusion protein was removed. This suggests that GST may have prevented *Ms. trichosporium* OB3b recombinant protein B from forming a fully functional sMMO complex. Fox *et al.* (1991) demonstrated that protein B from *Ms. trichosporium* OB3b binds to the α subunit of the hydroxylase component. It is unknown whether the same scenario exists in *Mc. capsulatus* (Bath). A reason for the lack of activity of GST-MTB could have been that the conformation of the protein was such that the binding site of protein B with the *Mc. capsulatus* (Bath) hydroxylase was occluded such that a fully functional sMMO complex was not formed. In contrast, the conformation of GST-MMOB must have been such that the binding site of protein B from *Mc. capsulatus* (Bath) to the hydroxylase was not obscured since this fusion protein was capable of forming a fully functional sMMO complex.

The question of the function of *orfY* is intriguing and since the cloning and DNA sequencing of the sMMO operon from *Mc. capsulatus* (Bath) (Stainthorpe *et al.*, 1989, 1990) there have been no investigations reported to elucidate its function in methane oxidation. The *orfY* DNA and amino acid sequences have no detectable homology with any sequences in the GenBank database (McDonald *et al.*, 1997). Experiments with anti-OrfY antibody did not succeed in determining whether *orfY* was translated in methanotrophs. In *E. coli*, weak cross-reactivity with a protein of

approximately 14 kDa was identified in *E. coli* BL21(DE3) [pT7-5sMMO]. Western blotting with sMMO and pMMO expressing methanotrophs identified cross-reactivity with *Mc. capsulatus* (Bath) proteins only. This occurred with proteins of greater than 40 kDa and so the 14 kDa OrfY protein was not identified. This suggested that *orfY* was not translated in *Mc. capsulatus* (Bath) or that translation did occur but the protein formed a complex with another polypeptide. Improved results with this work could have been obtained if: (1) the antibody had been raised to recombinant OrfY (and not GST-OrfY); (2) the antibody had been purified prior to use (Harlow and Lane, 1988); (3) a protein detection method more sensitive than the horseradish peroxidase system was used, such as the amplified detection system (Turner, 1983). To investigate further the role of *orfY, lacZ* transcriptional and translational reporter gene fusions are being constructed for use in methanotrophs (S. Harris and J. C. Murrell, personal communication). Another experiment that would improve our understanding of the role of *orfY* would be the use of marker exchange mutagenesis, to inactivate *orfY* by insertion of an antibiotic resistance cassette. This technique has been used to inactive the *mmoX* gene of *Ms. trichosporium* OB3b (Martin and Murrell, 1994). In a similar manner, it would be of great interest to inactivate *orfY*, to establish if functional expression of sMMO was retained or lost.

One of the limitations with the pGEX system was that thrombin was used to cleave GST from the protein of interest. This requires incubation of the protein with thrombin at room temperature for 5 to 60 min (Smith and Johnson, 1988). If the recombinant protein was unstable at this temperature then the problem could be made worse with this system of cleavage. A clear example, was the purification of recombinant protein B of *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b

(Chapter 6). Also, the amount of protease which can be used is limited by the need to restrict the amount of contaminating protein to a minimum. This can often lead to inefficient cleavage of the fusion protein. Efficient and rapid affinity purification of proteins has been reported using recombinant fusion proteases (Walker *et al.*, 1994). In this system, a highly active GST-$3C^{pro}$ 'fusion protease' encoded by the genome of a human rhinovirus was constructed using a GST affinity tail. This protease had a much greater specificity of proteolytic cleavage and complete removal of the protease and affinity tail in one step was possible using affinity chromatography. In addition, any amount of fusion protease could be used to ensure efficient cleavage and the fusion proteases were active at 4 °C. This system could overcome the limitations with the pGEX system but would require manipulation of the cleavage site from recognition by thrombin to $3C^{pro}$.

Recently, an alternative to the GST system has been developed by New England Biolabs®. The Impact I™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system utilises an intein from the *Saccharomyces cerevisiae VMA1* gene (Chong *et al.*, 1996). The intein undergoes a self-cleavage reaction at its N-terminus at low temperatures in the presence of thiols such as DTT, β-mercaptoethanol or cysteine. DNA encoding a 5 kDa chitin binding domain from *Bacillus circulans* has been added to the C-terminus of the intein for affinity purification of the fusion protein. Consequently, a target protein-intein-chitin binding domain fusion is constructed which can be bound to a chitin bead column. Cleavage of the recombinant protein is then achieved by the addition of DTT at 4 °C, to elute the native target protein. This overcomes the problems of performing the procedure at room temperature and does not require cleavage with a protease. In addition the C-

terminus of the target protein is used to fuse it to the intein-chitin binding domain (compared to the N-terminus for the GST-fusion system) and there are no tagged amino acids left at the C-terminus of the protein following cleavage. This system would be especially useful in over-expressing protein B from *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b in *E. coli* and could also prove to be a more efficient affinity purification system in general.

CHAPTER 6

Inactivation of the regulatory protein B from *Methylococcus capsulatus* (Bath) can be overcome by a Gly to Gln modification

6.1 Introduction

Protein B is a single subunit protein of 16 kDa containing no metals, prosthetic groups, or cofactors (Green and Dalton, 1985; Liu and Lippard, 1991). Occasionally, protein B has been observed to have a molecular mass of 31 kDa by gel filtration, suggesting that it is capable of existing as a dimer in *Mc. capsulatus* (Bath) (H. Dalton, personal communication), *Ms. trichosporium* OB3b (Fox *et al.*, 1989) and *Methylocystis* sp. M (H. Uchiyama, personal communication). Protein B alters the structure of the hydroxylase (Fox *et al.*, 1991; Paulsen *et al.*, 1994; George *et al.*, 1996; Davydov *et al.*, 1997) in such a way that it accelerates and potentially regulates sMMO (Green and Dalton, 1985; Fox *et al.*, 1991; Liu and Lippard, 1991). This could be attributed to protein B causing a conformational change in the substrate-binding site, possibly by altering the coordination of one or more of the helices containing ligands to the di-iron centre (Rosenzweig *et al.*, 1993).

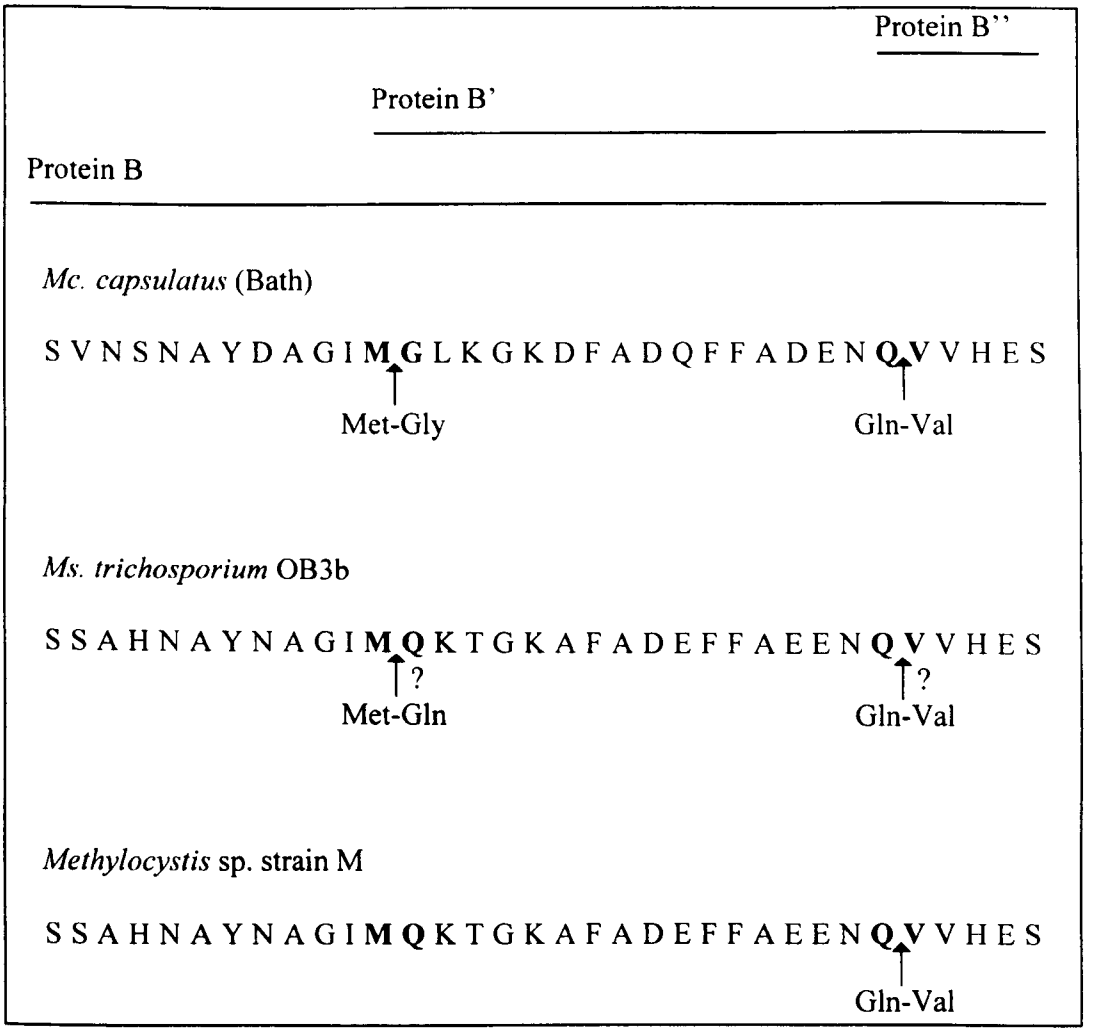
Purified protein B from *Mc. capsulatus* (Bath) is unstable and requires protease inhibitors to minimise its degradation to an inactive form, designated protein B' (Pilkington and Dalton, 1990). However, protein B always co-purifies with the truncated form B', at an approximate ratio of 1:1. Attempts to separate proteins B and B' in quantities suitable for enzymatic studies using a wide range of chromatographic techniques, including reverse phase and hydrophobic interaction chromatography, denaturation of the protein using urea followed by ion exchange chromatography, gel filtration and isoelectric focusing failed to separate the two forms of the protein (Bhambra, 1996). However, proteins B and B' can be separated as distinct bands on SDS-PAGE gels and N-terminal amino acid sequencing of the first 18 amino acids of proteins B and B' revealed that protein B lacked the N-terminal Met, predicted from

the DNA sequence, whereas the truncated form (B') lacked an additional 12 amino acids from the N-terminus (Bhambra, 1996). ESI-MS of a preparation of protein B (containing a mixture of B and B') from *Mc. capsulatus* (Bath) showed the presence of two main peaks with molecular masses of 15,852.6 ± 0.4 Da and 14,629.5 ± 0.3 Da, corresponding exactly to the predicted molecular masses of proteins B and B' respectively (Bhambra, 1996). This confirmed that protein B' arose from cleavage between Met12-Gly13 at the N-terminus of protein B (Figure 6.1) and was not due to a C-terminal cleavage as previously published (Pilkington *et al.*, 1990). Samples with increasing amounts of protein B' showed a decreasing activity in the standard sMMO assay and samples containing 100 % B' were totally inactive (Bhambra, 1996).

Fourier transform ion cyclotron resonance-mass spectrometry of a purified protein preparation containing proteins B and B', has identified a third protein designated B'' (A. Heck and H. Dalton, personal communication). Protein B'' accounted for about 20 % of the sample relative to protein B' and was thought to be a further breakdown product of protein B. Protein B'' was found to have a molecular mass of 13,273.6 ± 1.9 Da and sequence analysis indicated that this mass could result from the loss of 25 amino acids from the N-terminus of protein B, i.e. cleavage between Gln29-Val30 (Figure 6.1).

The gene encoding protein B from *Mc. capsulatus* (Bath) has been expressed in an active form in *E. coli* using a T7-RNA polymerase promoter expression system (West *et al.*, 1992). Enzyme activities of recombinant protein B in soluble extracts of *E. coli* K38 were comparable to the wild-type purified protein. Examination of soluble extracts and subsequent Western blotting with anti-serum against protein B of *Mc. capsulatus* (Bath) indicated that cleavage of protein B had occurred, since protein

Figure 6.1: Cleavage sites of the regulatory protein B from three methanotrophs.



The first 34 N-terminal amino acid residues are shown. ?, not known whether cleavage occurs.

B' was present (West *et al.*, 1992).

The truncation of protein B to B' represents a fundamental problem and poses many difficulties when performing biochemical analysis on protein B since it is readily degraded to an inactive protein. In addition, techniques such as X-ray crystallography are impossible to perform on protein B because of the inherent instability of the protein. Protein B purified from *Ms. trichosporium* OB3b has a specific activity of 11,200 nmol min^{-1} mg $protein^{-1}$ (Fox *et al.*, 1989), in comparison

to protein B from *Mc. capsulatus* (Bath) which has a specific activity of 7,289 nmol min^{-1} mg $protein^{-1}$ (Pilkington and Dalton, 1990). This 35 % higher activity of protein B from *Ms. trichosporium* OB3b could be attributed to the lack of truncation of protein B to an inactive form. Analysis of the amino acid sequences of protein B from these two organisms showed that the Met12-Gly13 cleavage site in *Mc. capsulatus* (Bath) was replaced by a Met12-Gln13 site in *Ms. trichosporium* OB3b (Figure 6.1) and there have been no reports of protein B' formation in this organism. Additional evidence for the lack of protein B' in *Ms. trichosporium* OB3b is that the same Met12-Gln13 site is present in *Methylocystis* sp. M and protein B' is not found, although Gln29-Val30 is cleaved (H. Uchiyama, personal communication).

The aim of the work described in this Chapter was to overcome the truncation of protein B from *Mc. capsulatus* (Bath), by first assessing the stability of recombinant protein B from *E. coli*, followed by site-directed mutagenesis to establish whether the Gly13 to Gln13 modification could overcome protein B' formation in *Mc. capsulatus* (Bath).

6.2 Heterologous expression of protein B

To determine whether protein B was cleaved by a protease common to both *Mc. capsulatus* (Bath) and *E. coli*, protease deficient *E. coli* strains were used. pEB51 (West *et al.*, 1992) consisting of a 1.7 kb *Pst*I fragment containing *mmo*B from *Mc. capsulatus* (Bath) in pT7-5, was electroporated into four different protease-deficient *E. coli* strains, BL21(DE3) (Studier and Moffatt, 1986), AD202 (Nakano *et al.*, 1994), UT5600 (McIntosh *et al.*, 1979) and SG20252 (Trisler and Gottesman, 1994). To over-express the *mmoB* gene in cells other than *E. coli* BL21(DE3), pGP1-2, which

contains the T7-RNA polymerase gene *1* was used (Tabor and Richardson, 1985). SDS-PAGE of soluble extracts followed by Western blotting with anti-serum against protein B from *Mc. capsulatus* (Bath) indicated that protein B' was present in soluble extracts from all *E. coli* strains tested.

E. coli BL21(DE3) is generally a good host for recombinant protein production because it is protease-deficient and contains a chromosomal copy of the T7 RNA polymerase gene (Studier and Moffatt, 1986) (Section 3.1). Induction of *E. coli* BL21(DE3) harboring pEB51 resulted in the expression of protein B to about 15 % of the total cell protein as estimated by SDS-PAGE. Phase contrast microscopy was used to identify inclusion bodies, suggesting that some of the recombinant protein B was being over-expressed as insoluble protein. Therefore, a variety of growth conditions were tested (Table 6.1) to maximise the amount of soluble recombinant protein. The concentration of soluble protein in batch culture was maximised by inducing expression with 0.5 mM IPTG at a growth temperature of 30 °C (Figure 6.2a). These growth conditions resulted in less than 1 % of the cells in the culture containing inclusion bodies, as estimated by phase contrast microscopy. No increase in expression level of protein B was seen after 2 h of IPTG induction by SDS-PAGE of whole cell lysates (Figure 6.2a) and so cells were harvested after this time. Subsequent work, in collaboration with Dr. A. Bhambra (University of Warwick, UK), to purify recombinant protein B from *E. coli* BL21(DE3) as described previously (Pilkington and Dalton, 1990), resulted in a preparation of recombinant protein B that was predominately protein B' as determined by SDS-PAGE and ESI-MS. The specific activity was 970 nmol min^{-1} mg $protein^{-1}$ which is very low when compared to wild-type enzyme activities of 7,289 nmol min^{-1} mg $protein^{-1}$ (Pilkington

Table 6.1: Optimisation of growth conditions for expression of soluble protein B in *E. coli* BL21(DE3).

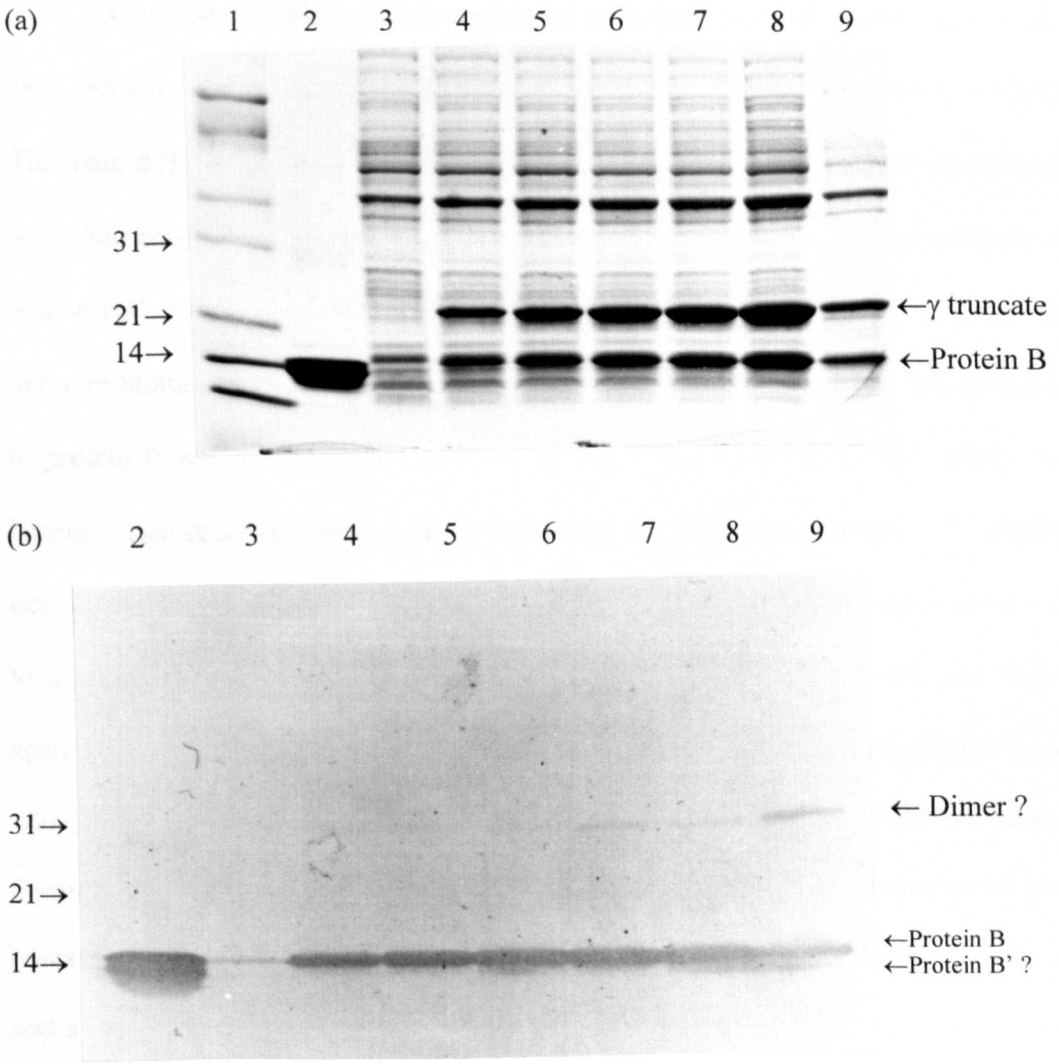
IPTG concentration (mM)	Growth temperature (°C)	Number of cells containing inclusion bodies after 5 h induction (%)
1	37	30
0.5	37	15
1	30	10
0.5	30	1

and Dalton, 1990) and the enzyme activities of recombinant protein B from *E. coli* K38 (West *et. al.*, 1992). Recombinant protein B from *E. coli* BL21(DE3) appeared less stable than protein B from *E. coli* K38 and *Mc. capsulatus* (Bath). Protein B is cleaved to inactive protein B' at identical residues in both *Mc. capsulatus* (Bath) and *E. coli*. If a protease is responsible for this cleavage, then it is common to both of these organisms. Due to the lack of success with this purification protocol, an alternative purification method was devised (Section 6.5).

6.3 Does the truncation of protein B occur during cell lysis?

The truncation of protein B occurred as soon as cells were lysed and soluble extracts prepared (Section 6.2). This work focused on whether truncated forms of protein B could be identified in whole cells of *E. coli* expressing recombinant protein B or *Mc. capsulatus* (Bath) expressing sMMO. Whole cell lysates of *E. coli* BL21

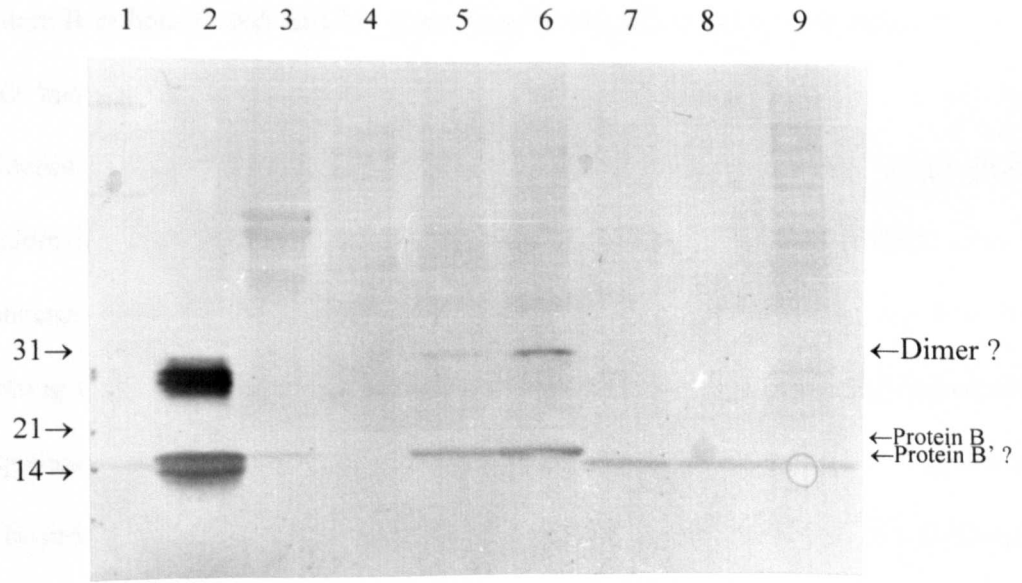
Figure 6.2: (a) SDS-PAGE and (b) Western blotting of T7 expression of protein B in whole cells of *E. coli* BL21(DE3). Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, pure protein B from *Mc. capsulatus* (Bath) (15 μg); lanes 3 to 9, whole cell lysates (approximately 30 μg) 0, 1, 2, 3, 4, 5 and 24 hr after induction with IPTG. Additional polypeptide in Figure 6.2a at 21.5 kDa is the γ-truncate of the hydroxylase (West *et al.*, 1992).



(DE3) [pEB51] were analysed by SDS-PAGE, followed by Western blotting with anti-serum against protein B from *Mc. capsulatus* (Bath) (Figure 6.2b). At 0 h induction, a basal level of recombinant protein B expression was seen which would be expected due to the leaky control of T7 expression in *E. coli* BL21(DE3). After 5 h, a single band corresponding to protein B was identified and possibly a small amount of a lower molecular mass truncated protein. However, at 24 h after IPTG induction, a truncated protein was identified at a ratio of about 1:5 relative to protein B. In addition a polypeptide of about 31 kDa that cross-reacted with the anti-serum against protein B was also observed.

A similar experiment was performed with wild-type *Mc. capsulatus* (Bath) cells (provided by R. Titmus, University of Warwick, UK) which expressed sMMO. The main difference in this experiment was that a stock of frozen cells were used and so a time course of protein B expression during growth was not performed. Whole cell lysates and soluble extracts were analysed by SDS-PAGE followed by Western blotting (Figure 6.3). For the whole cell lysates, a single band corresponding to protein B was identified and similarly to the Western blot for *E. coli* whole cell lysates, cross-reactivity with a protein of about 31 kDa was observed. In soluble extracts prepared from the same stock of whole cells, complete truncation of protein B to a lower molecular mass protein was seen which cross-reacted with anti-serum against protein B. It could be argued that this apparent decrease in molecular mass was due to the samples from the whole cell lysates and soluble extracts migrating through the polyacrylamide gel at slightly different rates. This experiment was repeated, and the Western blot this time showed a band corresponding to protein B and a much more intense band with a lower molecular mass that cross-reacted with

Figure 6.3: Whole cell lysates and soluble extracts of *Mc. capsulatus* (Bath) probed with anti-serum against protein B. Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, pure protein B from *Mc. capsulatus* (Bath) (15 μg); lanes 3 to 6, approximately 5, 10, 20 and 40 μg whole cell lysates; lanes 7 to 9, 5, 10 and 20 μg soluble extract.



anti-serum against protein B from *Mc. capsulatus* (Bath). This confirmed that the apparent decrease in molecular mass shown in the soluble extracts of Figure 6.3 was not due to retardation of the whole cell lysate samples through the polyacrylamide gel.

Protein B' was not identified in whole cells of *Mc. capsulatus* (Bath) expressing sMMO in continuous culture in the presence of methane. A lower molecular mass truncated form of protein B from *Mc. capsulatus* (Bath) was only identified in crude cell extracts. In contrast, a truncated form of protein B was identified in whole cells of *E. coli* after 24 h induction with IPTG. It was also interesting to note the cross-reactivity of a 31 kDa protein with the anti-serum against protein B in both *E. coli* and *Mc. capsulatus* (Bath) whole cells. The major problem with this technique for investigating protein B truncation was that there was no evidence to suggest that the lower molecular mass truncates had not arisen from random degradation of the recombinant protein. Therefore, specific Met12-Gly13 truncates of protein B could not be identified in whole cells using this Western blotting technique. However, this problem could be overcome by using N-terminal sequencing. If a protease was involved in the truncation of protein B, then it appeared to be most active when whole cells were broken suggesting that it may be membrane bound and released only upon cell lysis.

6.4 Protease inhibition of protein B truncation

It has been reported previously that a wide range of protease inhibitors do not inhibit protein B' formation in *Mc. capsulatus* (Bath) (Bhambra, 1996). The effects of the following protease inhibitors on protein B truncation were determined: phenylmethylsulfonylfluoride (PMSF) (1 mM); benzamidine (1 mM); pefabloc SC

(4 mM) and α-2-macroglobulin (2 μM). Protease inhibitors were added to 25 mM MOPS buffer pH 7.0, which was used to resuspend whole cells of *E. coli* BL21(DE3) [pEB51] and *Mc. capsulatus* (Bath) expressing sMMO. Soluble extracts were prepared, separated by SDS-PAGE and Western blotted with anti-serum against protein B from *Mc. capsulatus* (Bath). In all cases, protein B and a protein of lower molecular mass that cross-reacted with anti-serum against protein B from *Mc. capsulatus* (Bath) were identified. The lower molecular mass protein was present at a ratio of about 2:1 with respect to protein B, in all cases. This work confirmed that these protease inhibitors had no effect on minimising protein B truncation and were consistent with previous work by Bhambra (1996) where 1 mM PMSF, 1 mM EDTA, 1 mM benzamidine, 5 μM soybean trypsin inhibitor, 28 μM trans-epoxysuccinyl-*L*-leucylamide-(4-guanidine) butane, 4 mM pefabloc SC and 1 μM pepstatin A also had no effect on reducing protein B truncation.

Although it was impossible to use every protease inhibitor to determine its effect on protein B, it is clear that the compounds tested did not overcome protein B' formation. These results, in conjunction with those obtained in Section 6.2, also lend support to the hypothesis that protein B truncation is caused by autocatalysis rather than a protease.

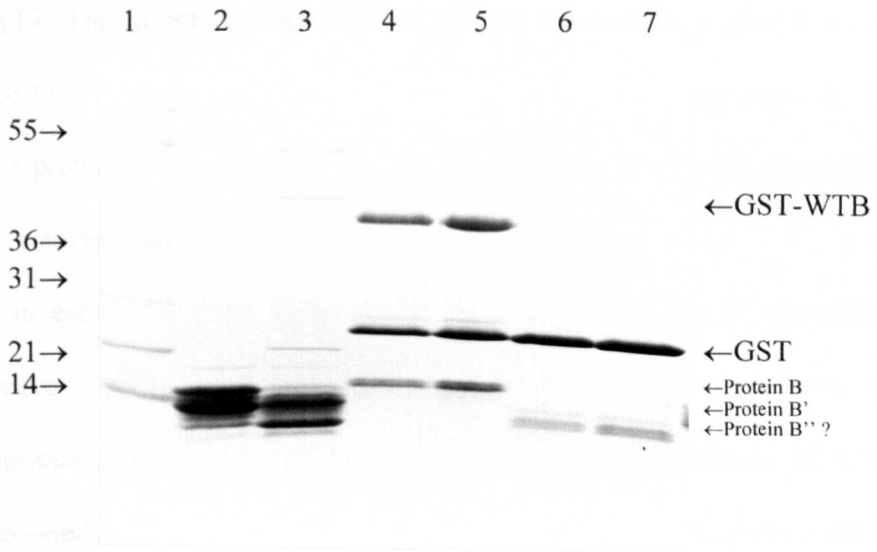
6.5 Affinity purification of protein B

It was apparent that recombinant protein B must be rapidly purified from crude extracts, due to the low activities and rapid degradation of recombinant protein B to inactive protein B'. To achieve this, a GST fusion protein was constructed with *mmo*B, forming pGEX-WTB in *E. coli* AD202, as described in detail in Section 5.3.1.

For the purposes of this work, it was of interest to determine the stability of GST-WTB when incubated at 20 °C and to establish if protein B was still cleaved at Met12-Gly13 whilst fused to GST. GST-WTB was purified using affinity chromatography (Section 2.12.1) and immediately after purification, the fusion protein was frozen in liquid nitrogen and stored at -70 °C, to minimise any degradation. To determine if the fusion protein was cleaved at Met12-Gly13 in the absence of thrombin, an aliquot of GST-WTB was incubated at 20 °C for 3 days. Samples were then analysed using SDS-PAGE (Figure 6.4). Immediately after purification, the 43 kDa GST-WTB fusion protein had been partially cleaved to give rise to two additional proteins at 27 kDa and 16 kDa. Western blotting with anti-serum against protein B from *Mc. capsulatus* (Bath) showed that the 43 kDa and 16 kDa proteins cross-reacted with the anti-serum. Further incubation of GST-WTB for 3 days at 20 °C showed complete loss of the 43 kDa protein, whilst the 27 kDa protein band had increased in intensity and the protein at 16 kDa was no longer visible. Instead a 15 kDa protein that cross-reacted with anti-serum against protein B from *Mc. capsulatus* (Bath) was present which suggested that complete degradation of protein B had occurred (which was consistent with the observed loss of enzyme activity). Therefore, by using SDS-PAGE and Western blotting it was shown that GST-WTB was subject to truncation even in the absence of thrombin.

To assess the cleavage of this protein more precisely, ESI-MS was used to obtain exact molecular masses for the various truncated proteins. However, due to the problem encountered with respect to an anomalously high molecular mass for GST being consistently obtained (Section 5.3.1), this proved difficult. ESI-MS of the sample used in lanes 5 and 7 of Figure 6.4 identified a protein with a molecular mass

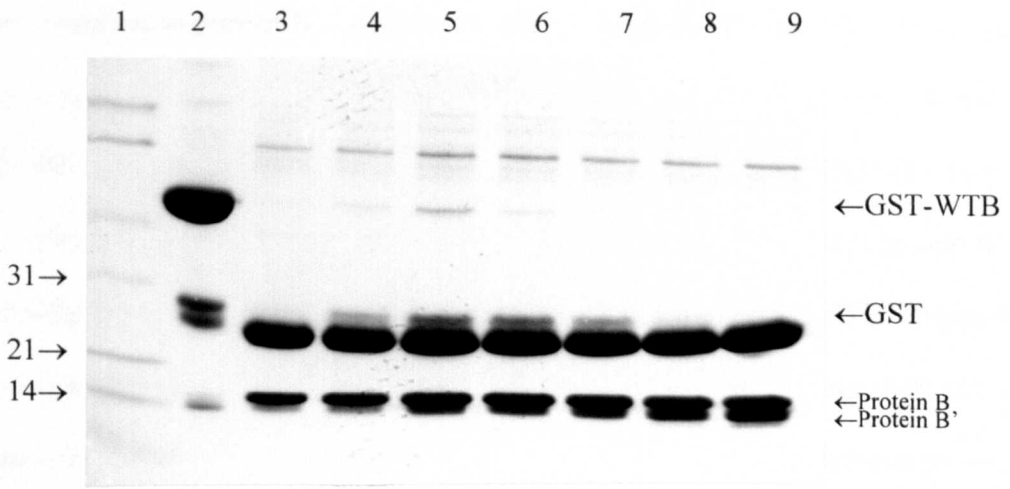
Figure 6.4: SDS-PAGE of GST-WTB incubated at 20 °C for 3 days. Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, protein B (10 μg); lane 3, protein B (10 μg) incubated at 20 °C for 3 days; lane 4, pGEX-WTB (10 μg); lane 5, pGEX-WTB (20 μg); lane 6, pGEX-WTB (10 μg) incubated at 20 °C for 3 days; lane 7, pGEX-WTB (20 μg) incubated at 20 °C for 3 days.



of 14,627.25 Da (protein B'), but no other ESI-MS peaks corresponded to the predicted molecular masses for the other proteins. This was expected owing to the incorrect molecular mass obtained for GST which would also make subsequent calculations for other proteins fused to GST incorrect too. However, protein B was not identified in the sample, suggesting that cleavage between Met12-Gly13 was responsible for the truncation of GST-WTB and that the first 12 amino acids at the N-terminus of protein B remained tagged to GST. Therefore, although the N-terminus of protein B was "protected" by fusing it to GST, cleavage still occurred between Met12-Gly13. During translation of GST-WTB, the N-terminus of protein B may be expected to fold within the fusion protein. Since Met12-Gly13 was cleaved, the N-terminus of protein B must somehow be exposed for it to be readily susceptible to proteolytic cleavage, whether by a protease or autocatalysis.

To investigate further the formation of recombinant protein B`, thrombin was used to cleave WTB from GST. Initially after cleavage, GST was not removed by affinity chromatography to reduce the amount of time for degradation of WTB to occur. The stability of WTB was investigated by incubating separated GST and WTB at 20 °C for up to 3 h. Aliquots were then analysed by SDS-PAGE to determine the extent of protein B' formation (Figure 6.5). Protein B' appeared within 20 min at 20 °C and by 3 h, greater than 60 % of WTB had degraded to protein B'. Molecular masses were confirmed by ESI-MS. When purified protein B from *Mc. capsulatus* (Bath) was incubated for the same amount of time both in the presence and absence of thrombin, no difference in the degradation of protein B was seen, which proved that thrombin was not responsible for causing protein B truncation.

Figure 6.5: SDS-PAGE of GST-WTB incubated at 20 °C for 3 h, after cleavage with thrombin. Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, GST-WTB (20 μg) without thrombin; lanes 3 to 9, GST-WTB (20 μg) 5, 10, 20, 30, 60, 120 and 180 min respectively, after addition of thrombin.

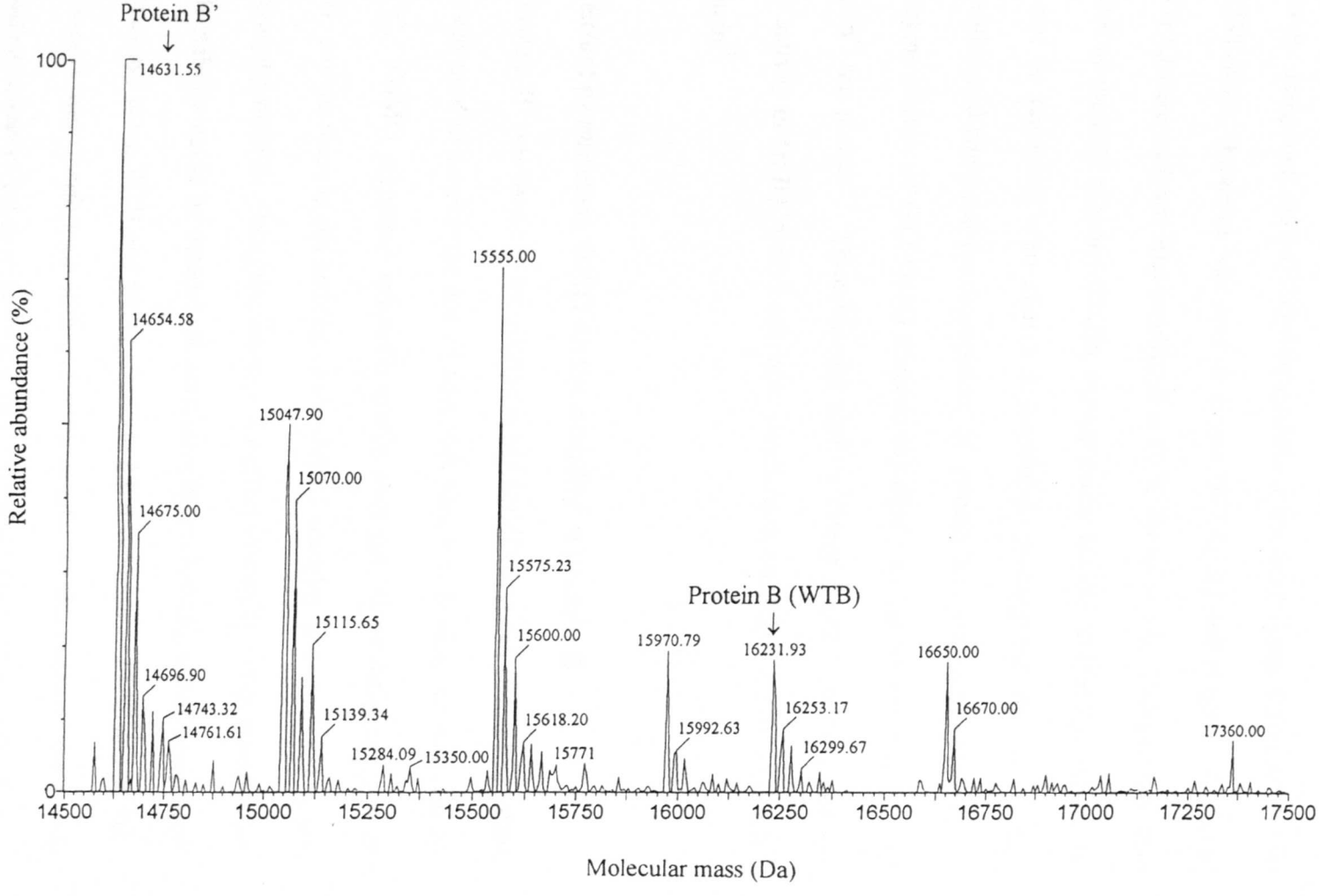


The stability of WTB was also investigated after removing GST. WTB was incubated at 20 °C for 24 h in 25 mM MOPS, pH 7.0 and aliquots taken during the time course were analysed using SDS-PAGE (data not shown). Immediately after purification, WTB and a lower molecular mass protein of about 14 kDa were identified. These proteins cross-reacted with anti-serum against protein B from *Mc. capsulatus* (Bath) and were likely to be proteins B and B'. Continued incubation resulted in complete loss of WTB by 10 h and an increase in intensity of the band corresponding to protein B'. Another protein of about 13 kDa (possibly protein B'') that cross-reacted with anti-serum against protein B was also observed. A typical ESI-MS spectrum of a purified preparation of WTB is shown in Figure 6.6. Protein B'' was not detected but the spectrum does confirm the large amount of protein B' in the sample, which is present at a ratio of about 5:1 with respect to WTB. When this was compared to the ESI-MS analysis of protein B from *Mc. capsulatus* (Bath), the ratio of protein B' to B was about 1:1 (Bhambra, 1996). This indicated that protein B from *E. coli* was less stable than protein B from *Mc. capsulatus* (Bath). Since there have been no reports of protein B' formation in *Ms. trichosporium* OB3b, the Met12-Gly13 cleavage site of *Mc. capsulatus* (Bath) was mutated to the corresponding Met12-Gln13 sequence of *Ms. trichosporium* OB3b by site-directed mutagenesis in an attempt to preclude protein B' formation.

6.6 Site-directed mutagenesis of protein B

The Gly13 to Gln13 mutation was introduced into pGEX-WTB using the Unique Site Elimination kit (Pharmacia Biotech) (Section 2.10). A mutagenesis efficiency of 65 % was obtained for the control reaction in comparison to a protein B

Figure 6.6: ESI-MS analysis of WTB.

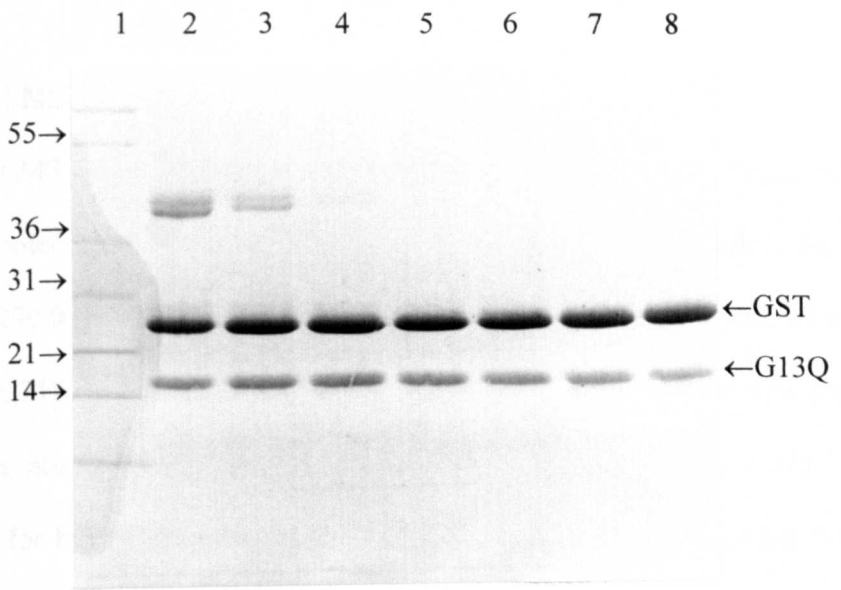


mutation efficiency of 5 %. The mutated gene was confirmed using DNA sequencing and the plasmid obtained was designated pGEX-G13Q. The stability of mutant fusion protein B, designated GST-G13Q was initially determined using SDS-PAGE. As with GST-WTB, thrombin was used to cleave GST-G13Q and aliquots of cleaved GST-G13Q were analysed after incubation at 20 °C for up to 3 h. Two proteins were identified at about 27 kDa and 16 kDa, corresponding to GST and G13Q. Protein B' could not be identified even after 3 h incubation, proving that the Gly to Gln modification had alleviated the degradation of protein B to protein B' (Figure 6.7). The enzyme activity of GST-G13Q immediately after purification was 6,200 ± 145 nmol min^{-1} mg $protein^{-1}$. Thrombin was used to cleave G13Q from GST and the enzyme activity of G13Q immediately after purification was 8,200 ± 230 nmol min^{-1} mg $protein^{-1}$.

6.7 Effect of mutation G13Q on the stability of protein B

Protein B' was found to be inactive in the sMMO complex (Bhambra, 1996) and this was used to monitor the degradation of protein B following incubation at 20 and 37 °C. sMMO propylene oxidation assays were performed using samples of protein B; any decrease in the activity of the sMMO complex was attributed to the inactivation of protein B. Using this strategy, the effect of the Gly to Gln mutation on protein B stability could be determined accurately by performing sMMO propylene oxidation assays using WTB and G13Q after 0 to 8 h incubation at 20 °C and 37 °C. Enzyme assays were performed with and without GST to determine if the fused protein had any direct effect on enzyme stability. At 20 °C, GST-WTB retained 61 ± 4 % activity after 8 h, compared to GST-G13Q which retained 98 ± 3 % activity.

Figure 6.7: SDS-PAGE of GST-G13Q incubated at 20 °C for 3 h, after cleavage with thrombin. Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2 to 8, GST-G13Q (20 μg) 5, 10, 20, 30, 60, 120 and 180 min respectively, after addition of thrombin.

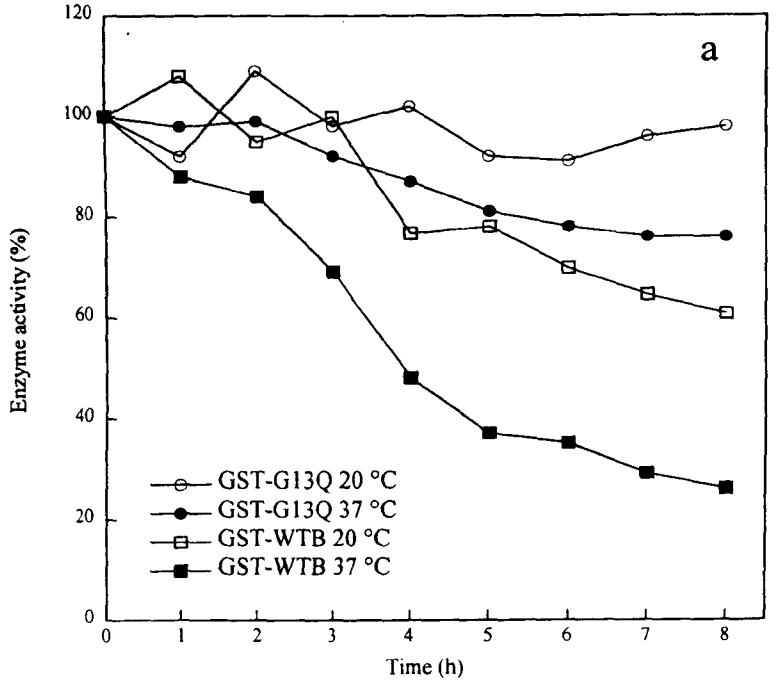


GST-WTB retained 26 ± 5 % activity and GST-G13Q retained 76 ± 4 % activity after 8 h at 37 °C (Figure 6.8a). Stability assays were also performed with WTB and G13Q. At 20 °C, WTB retained 46 ± 2 % activity after 8 h, compared to G13Q which retained 57 ± 3 % activity. At 37 °C, WTB retained 20 ± 4 % activity after 8 h, compared to G13Q which retained 32 % ± 3 % activity (Figure 6.8b). Therefore, GST-G13Q was approximately 60 % more stable than GST-WTB and G13Q was approximately 20 % more stable than WTB. No decrease in the activity of the hydroxylase or reductase was detected during the time taken to perform the assays.

6.8 ESI-MS of G13Q

ESI-MS analysis of a pure preparation of G13Q is shown in Figure 6.9. The expected molecular mass of G13Q was 16,300.1 Da and the main peak in the sample was at 16,299.0 Da and was therefore assigned as G13Q. A protein with a molecular mass of 16,717 Da which accounted for about 35 % of the sample with respect to G13Q was also identified in the sample. This molecular mass could be only accounted for by assuming that it was G13Q with two molecules of MOPS cation bound, which had each lost a sodium anion. The phenomenon of the loss of sodium anions from MOPS is though to occur because the positively charged protein replaces the positively charged sodium salt bound to a molecule of MOPS. If this was the case then the expected molecular mass would be 16,715.4 Da, in good agreement with the experimentally determined mass. A protein with a molecular mass of 16,229 Da that accounted for about 15 % of the sample relative to G13Q was also identified. A cleavage product of G13Q could not be assigned to this molecular mass, however, the expected molecular mass of WTB was calculated as 16,229.02 Da (Section 5.3.1).

Figure 6.8: sMMO propylene oxidation stability assays of (a) GST-WTB and GST-G13Q (b) WTB and G13Q pre-incubated for 8 h at 20 and 37 °C. sMMO propylene oxidation assays were performed by adding saturating amounts of hydroxylase and reductase to 8 μM protein B. Enzyme activity is shown as the percentage of activity remaining after incubation (compared to activity at 0 h). 100 % activity represents 5,700 ± 340 nmol min^{-1} mg $protein^{-1}$ (GST-WTB); 6,200 ± 145 nmol min^{-1} mg $protein^{-1}$ (GST-G13Q); 8,800 ± 410 nmol min^{-1} mg $protein^{-1}$ (WTB) and 8,200 ± 230 nmol min^{-1} mg $protein^{-1}$ (G13Q). Standard error was 6 %.



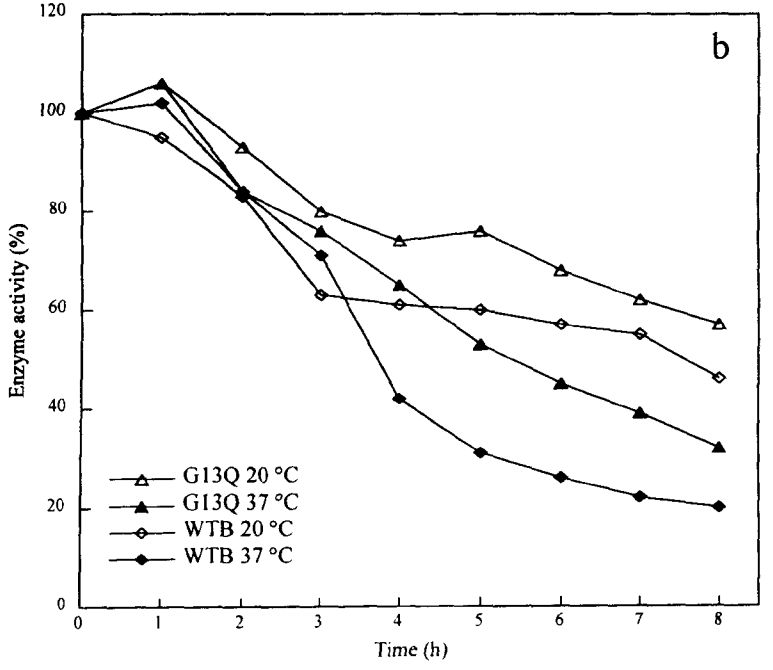
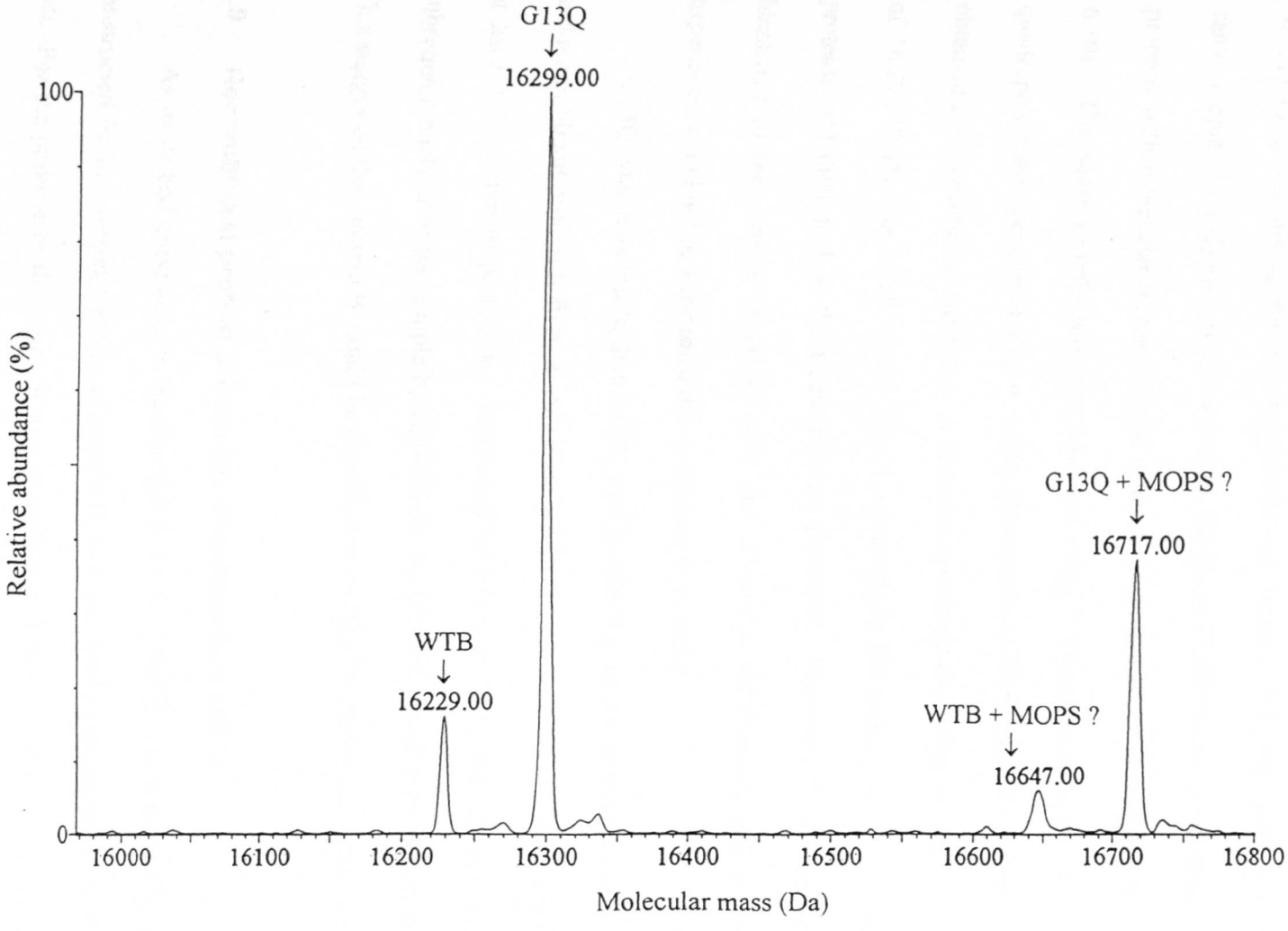


Figure 6.9: ESI-MS analysis of purified G13Q.



This suggested that purified WTB was present in the sample. In addition, the peak at 16,647 Da could be accounted for by assuming that two molecules of MOPS (minus sodium salt) were bound to the protein. The expected molecular mass was 16,645.4 Da, suggesting that this hypothesis was correct. ESI-MS analysis of the same sample of G13Q on a mass scale of 11,500 Da to 17,000 Da did not identify any proteins with molecular masses similar to those of protein B' or protein B'' (Figure 6.10). The same sample was also analysed using a Micromass Quattro II triple quadrupole mass spectrometer by A. Millar (University of Warwick, UK) and the data obtained are presented in Table 6.2. A suitable experiment to confirm that the protein of 16,229.02 Da was WTB would have been to digest the protein with a suitable protease and then analyse the fragment sizes produced. However, WTB was only identified in the sample of G13Q once out of several purifications and so this experiment could not be performed due to the lack of material.

G13Q was more stable than WTB, pure protein B from *Mc. capsulatus* (Bath) and recombinant protein B from *E. coli* all of which contained protein B' at a ratio of at least 1:1 relative to protein B. Incubation of G13Q at 20 °C for 120 min and subsequent analysis of the sample by ESI-MS did confirm the presence of protein B'. This suggested that protein B' could be identified in G13Q after prolonged incubation.

6.9 Recombinant protein B from *Ms. trichosporium* OB3b

As described previously in Section 5.3.2, the GST-MTB fusion protein was constructed for the over-expression of protein B from *Ms. trichosporium* OB3b in *E. coli*. For the purpose of this work, it was of interest to determine whether truncated

Figure 6.10: ESI-MS analysis of purified G13Q on a mass scale of 11,500 Da to 17,000 Da.

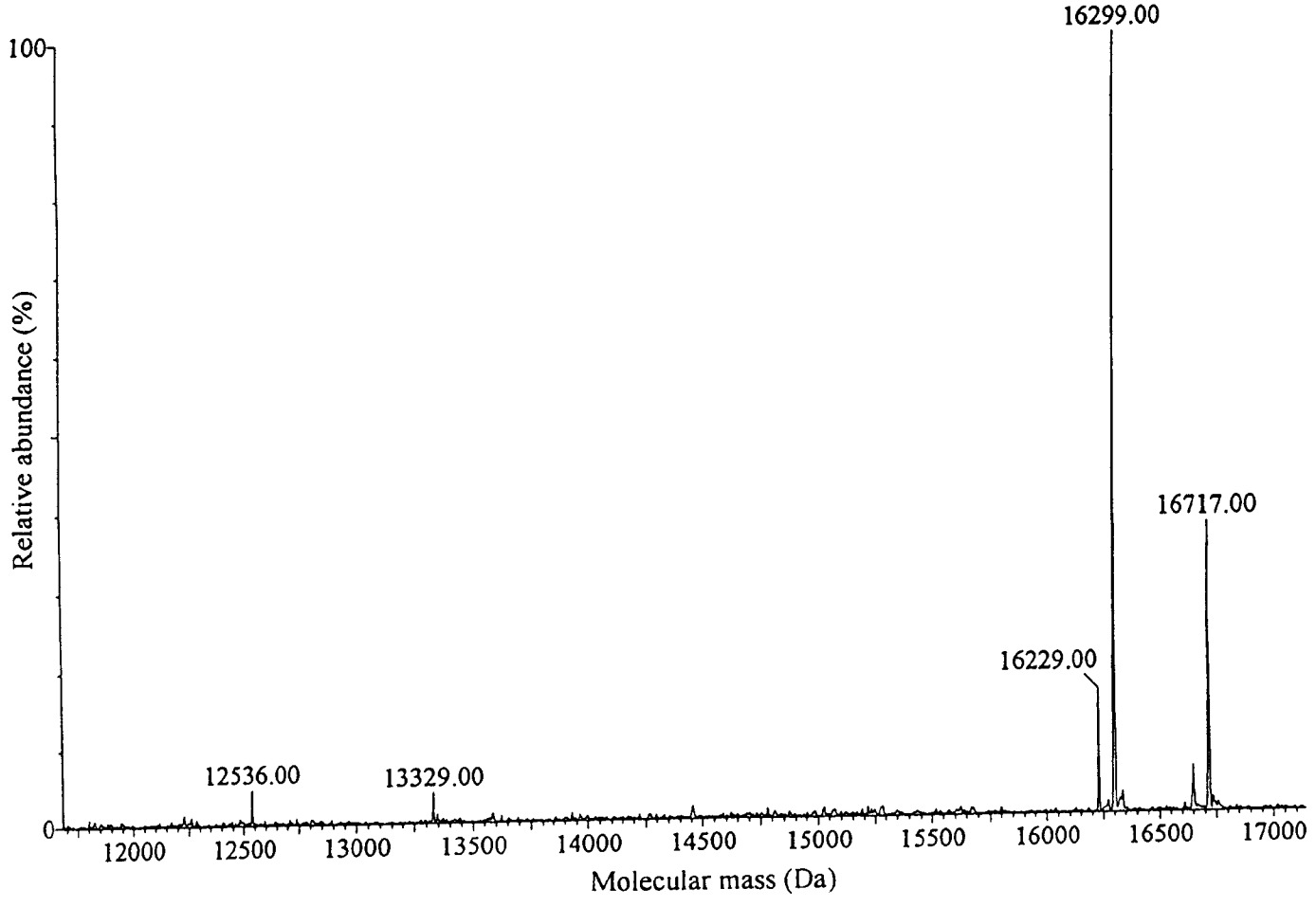


Table 6.2: ESI-MS analysis of pure G13Q.

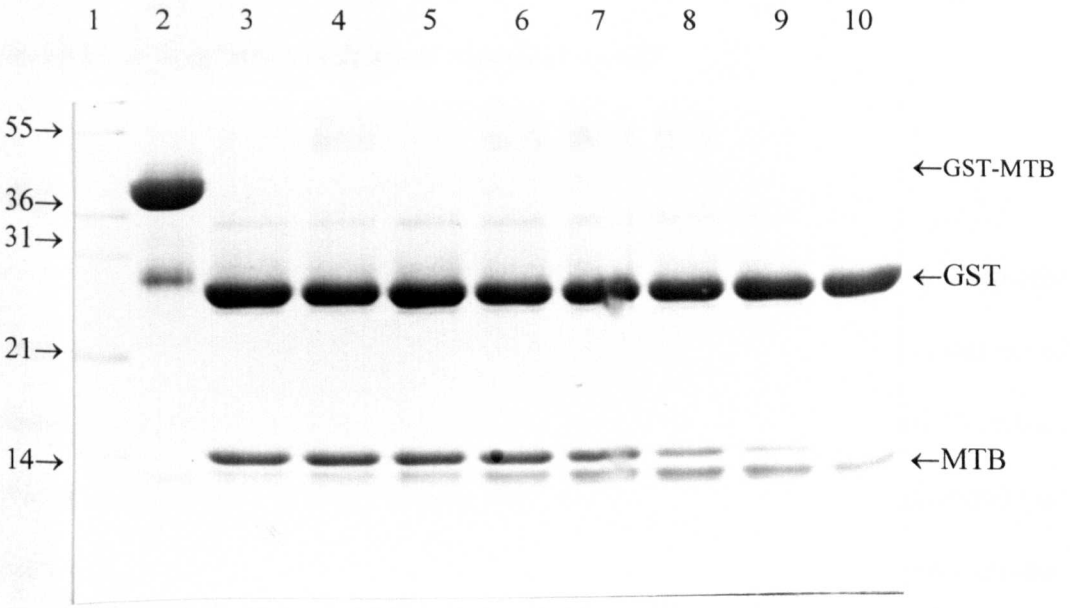
Fisons molecular mass (Da)[1]	Micromass molecular mass (Da)[2]	Calculated molecular mass (Da)	Protein
16,227.0	16,228.3	16,229.02	WTB
16,299.3	16,299.6	16,300.1	G13Q
16,647.0	16,645.2	16.645.4	WTB + MOPS
16,717.0	16,715.9	16,715.4	G13Q + MOPS

[1] Molecular mass was determined using a Fisons Quattro II triple quadrupole mass spectrometer (VG Biotech).

[2] Molecular mass was determined using a Micromass Quattro II triple quadrupole mass spectrometer.

forms of protein B could be identified, especially with respect to cleavage between Met12-Gln13 and Gln29-Val30. There have been no reports of the truncation of protein B in this organism and the requirement for protease inhibitors was not reported during the purification of this protein (Fox *et al.*, 1989). ESI-MS analysis of G13Q has confirmed that protein B truncation does occur between Met12-Gln13, suggesting that protein B' formation may occur in *Ms. trichosporium* OB3b. The GST-MTB fusion protein was purified and then cleaved with thrombin to release MTB from the GST fusion protein. Following incubation at 20 °C for 3 h, aliquots were sampled for analysis by SDS-PAGE (Figure 6.11). Results indicated that cleavage of GST-MTB had occurred immediately after purification (in the absence of

thrombin). After 5 min **Figure 6.11: SDS-PAGE of GST-MTB incubated at 20 °C for 3 h, after cleavage with thrombin.** Lane 1, protein molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, GST-MTB (20 μg) without thrombin; lanes 3 to 10, GST-MTB (20 μg) 1, 5, 10, 20, 30, 60, 120 and 180 min respectively after addition of thrombin.



incubation in the presence of thrombin, two additional proteins with lower molecular masses than MTB were visible and by 60 min, an additional protein with a lower molecular mass than the other two proteins was identified. Anti-serum against protein B from *Ms. trichosporium* OB3b was not available for Western blotting experiments to confirm that the lower molecular mass proteins were truncated forms of MTB. However, it is highly likely that truncation of MTB was the cause for the additional lower molecular mass proteins. The evidence for this was provided by the clear disappearance of MTB during incubation at 20 °C and the concomitant increase in intensity of the proteins with lower molecular masses.

6.10 Discussion

It is still unclear if the formation of protein B' was the result of a separate protease-mediated cleavage, an autocatalytic cleavage process or a combination of these mechanisms. Cleavage of protein B occurs between residues 12 and 13 in both *Mc. capsulatus* (Bath) and the recombinant protein B in *E. coli*, which suggested that an autocatalytic process may have been occurring. Protein B' was still observed when *mmoB* was expressed in various strains of protease-deficient *E. coli*. Trypsin proteolysis of protein B analysed by N-terminal sequencing (D. D. S. Smith and H. Dalton, unpublished observations) showed that trypsin did not cleave at Met12-Gly13 and so a trypsin-like protease was not involved in the cleavage of protein B. Judging from Coomassie Brilliant Blue stained gels and ESI-MS of pure protein B from *Mc. capsulatus* (Bath) (Bhambra, 1996) there did not appear to be another enzymatic species present. Certainly if trace amounts of a protease responsible for protein B

cleavage were present, then the protease was not inhibited by any of the standard protease inhibitors tested here.

Protein B' can be detected in soluble extracts of *Mc. capsulatus* (Bath) and *E. coli*. Degradation of protein B has also been detected in whole cells of *E. coli* expressing protein B but whether this degradation was due to a specific Met12-Gly13 cleavage is unknown since ESI-MS or N-terminal sequencing were not used. SDS-PAGE and Western blotting of whole cells of *Mc. capsulatus* (Bath) have failed to identify a truncated form of protein B.

It has been suggested that protein B may exist as a dimer in *Ms. trichosporium* OB3b (Fox *et al.*, 1989) and *Methylocystis* sp. M (H. Uchiyama, personal communication) but as a single subunit protein in *Mc. capsulatus* (Bath) (Green and Dalton, 1985). Results from Western blots of whole cell lysates of *Mc. capsulatus* (Bath) expressing sMMO and *E. coli* expressing recombinant protein B indicated cross-reactivity with proteins of approximately 16 and 31 kDa. This could represent the monomeric and dimeric forms of protein B from *Mc. capsulatus* (Bath), suggesting that the boiling of whole cells in SDS-sample buffer prevented the disruption of the dimeric protein which disappeared upon preparation of soluble extracts. There is the possibility that the anti-serum against protein B cross-reacted non-specifically with a 31 kDa protein unrelated to protein B in the whole cell lysates. When the same experiment was performed with *E. coli* AD202 [pT7-5] cross-reactivity was not observed with the whole cell lysates, insoluble or soluble extracts confirming that non-specific cross-reactivity was an unlikely explanation. Also, it would be unlikely that the non-specific cross-reactivity of the antibody would be seen with a protein of the same molecular mass in both *Mc. capsulatus* (Bath) and *E. coli*.

The hypothesis that the dimeric form of protein B was not found in soluble extracts may also serve to explain why Green and Dalton (1985) only observed a single subunit size of 17 kDa by gel filtration.

Expression of protein B as a GST fusion protein resulted in preparations of protein B with low levels of B' formation as determined by ESI-MS and specific activities comparable, if not higher, than protein B purified from *Mc. capsulatus* (Bath). At the C-terminus of protein B, a Met130-Gly131 site is found but is not cleaved, perhaps because it remains folded within the protein. Similarly, inhibition of protein B degradation may be expected in purified preparations of GST-WTB if the N-terminus of protein B was folded within the fusion protein. However, GST-WTB was cleaved to release protein B', suggesting that the N-terminal region of protein B within the GST-fusion was more susceptible to degradation than the C-terminus. Alternatively, the N-terminal region of protein B may not have folded within the protein. Enzyme activity was observed for GST-WTB when pure hydroxylase and pure reductase were used to reconstitute sMMO. As soon as thrombin was added to release WTB from GST, protein B' was identified by SDS-PAGE. Alteration of Gly13 to Gln13 enhanced the stability of recombinant protein B preparations but continued incubation of G13Q at 20 or 37 °C did result in protein B' formation.

It has not been established why the 12 amino acids at the N-terminus of protein B play such a critical role in the activity of this protein. Binding constants determined using surface plasmon resonance, indicated that protein B' was able to bind to the hydroxylase and reductase components (Bhambra, 1996). Binding of protein B' to the hydroxylase was also confirmed using direct electrochemistry (Kazlauskaite *et al.*, 1996). Based upon energy minimisation calculations it has been

proposed by George *et al.* (1996) that sMMO has three substrate binding sites, a unique binding site (about 3 Å from the di-iron centre) and two further sites termed A and B. Since substrates with a volume greater than ~ 71 $Å^3$ cannot be accommodated at the unique binding site, they must bind at sites A or B which are about 14 Å from the di-iron centre. Thus a conformational change, possibly brought about by the regulatory protein B, must occur to allow substrates at sites A or B to become oxidised. Additional evidence obtained by Davydov *et al.* (1997) using radiolytic reduction, also showed that protein B caused a conformational change in the hydroxylase, prior to the reduction of the hydroxylase to the di-ferrous state. Therefore, loss of sMMO activity may be due to the inability of protein B' to cause a conformational change in the hydroxylase di-iron centre. Exactly how the 12 amino acids at the N-terminus of protein B interact with the hydroxylase to bring about a conformational change is unknown, although a vast amount of information could be obtained from an X-ray crystal structure of protein B and protein B bound to the hydroxylase. Attempts to determine the effect of recombinant protein B upon the X-ray crystal structure of the hydroxylase component from *Ms. trichosporium* OB3b were unsuccessful even with protein B present at a two-fold excess (Elango *et al.*, 1997). SDS-PAGE of washed crystals indicated that protein B was not present. To obtain crystals, the hydroxylase and recombinant protein B were stored at 23 °C for 16 days. Elango *et al.* (1997) failed to describe details about the source of the recombinant protein B used in their study. Consistent with the work outlined in this Chapter, storage of recombinant protein B from *Ms. trichosporium* OB3b for 16 days at 23 °C would result in its disappearance, due to the susceptibility of the protein to degradation. This therefore provides additional evidence that protein B from *Ms.*

trichosporium OB3b is susceptible to truncation and fully justifies the approach to improve the stability of protein B from *Mc. capsulatus* (Bath) prior to its crystallisation. The only conclusions that could be made by Elango *et al.* (1997) were that protein B had either a very subtle effect upon the hydroxylase that was not detected at 2.4 Å or (more likely) that the hydroxylase had returned to its native state.

In addition to overcoming protein B truncation for X-ray crystallography and other biochemical analysis, there is also interest in protein B' formation as a general regulatory mechanism for the sMMO complex. This may function by controlling the amount of active protein B in the cell and allowing the conservation of energy, by preventing wasteful oxidation of NADH in the absence of any substrate. Green and Dalton (1985) first proposed the hypothesis that uncoupling of sMMO could cause dissociation of protein B from the sMMO complex and that free protein B could then be turned over by proteases in the cell. *De novo* synthesis of protein B could then recouple the system. It was suggested that such a scenario may occur during growth on methanol, where accumulation of the toxic metabolite formaldehyde occurred only during growth using pMMO (Cornish *et al.*, 1984). It was suggested by Green and Dalton (1985) that formaldehyde accumulation did not occur when cells expressing sMMO were grown on methanol because uncoupling of sMMO would allow regulation of the levels of NADH and NAD^+ in the cell. Consequently, the generation of NAD^+ (the coenzyme for formaldehyde and formate dehydrogenase) would overcome formaldehyde toxicity.

Another example of regulation by protein cleavage is the SOS response in *E. coli*, which is induced following exposure to agents that damage DNA or interfere with DNA replication (Shingawa *et al.*, 1988). The SOS response increases the ability

of the cell to perform mutagenesis and requires the products of the *umuD*, *umuC* and *recA* genes. LexA regulates expression of these genes by binding to the operator region of *recA* and *umuDC* to repress transcription of the genes in uninduced cells. Exposure to DNA damaging agents, activates RecA to form RecA* which causes cleavage of LexA between Ala112-Gly113. This inactivation of LexA by proteolytic cleavage causes increased expression of SOS genes. In addition, RecA mediates cleavage of UmuD to UmuD* between Cys24-Gly25 leading to activation for its mutagenic function. Cleavage of UmuD is thought to occur when the protein is monomeric and formation of the dimer (which is not cleaved) may be another level of control for UmuD since auto-digestion is prevented (Peat *et al.*, 1996). Mutations of the RecA cleavage site caused a major reduction in the ability of UmuD to be active in UV mutagenesis (Nohmi *et al.*, 1988). Therefore the SOS response is regulated by RecA and UmuD mediated inactivation and activation.

Cleavage of protein B to B' may be due to activation of an autocatalytic process under certain conditions such as lack of substrate or NADH. Experiments are currently in progress to determine if environmental conditions will lead to the inactivation of protein B *in vivo* (A. Callaghan and H. Dalton, personal communication). Only then will the ultimate significance of the inactivation of protein B become clear.

CHAPTER 7

Expression of sMMO genes from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b in pMMO-only methanotrophs

7.1 Introduction

The lack of a suitable method to introduce DNA into methane-oxidising bacteria has been a major problem in the development of molecular genetic techniques for methanotrophs (Murrell, 1994). It has been reported that of ten obligate methanotrophs studied, plasmid DNA was identified in all strains except *Mc. capsulatus* (Bath) (Lidstrom and Wopat, 1984). Of these strains, *Ms. trichosporium* OB3b contained three plasmids of 186, 159 and 145 kb, *Mcy. parvus* OBBP contained two plasmids of 186 and 159 kb, whilst *Mm. album* BG8 contained one plasmid of 55 kb. The 55 kb plasmid of *Mm. album* BG8 was studied in further detail, however, due its large size and absence of selectable markers, no further developments have been reported with regard to its use as a cloning vector.

The first report of conjugal transfer into a methanotroph was by Warner *et al.* (1980). In this study, filter matings between *Pseudomonas aeruginosa* PA08 [R68.45] and *Ms. trichosporium* OB3b, using Km (50 μg ml^{-1}) as the selectable marker, resulted in the isolation of transconjugants at a frequency of transfer of 10^{-2} to 10^{-3} per donor. Subsequently, a conjugation system was developed for gene transfer into three different methanotrophs (Lidstrom *et al.*, 1984) using the cosmid cloning vector, pVK100 (Knauf and Nester, 1982) mobilised by pRK2013 (Figurski and Helinski, 1979). Transfer frequencies of 10^{-2} were obtained for *Mm. album* BG8 and *Methylocystis* POC but a lower frequency of 10^{-8} was reported for *Methylosinus* 6. McPheat *et al.* (1987a) transferred the plasmids RP4 and R300B from *E. coli* donors to *Mm. album* BG8 at frequencies of 2×10^{-3} and 1×10^{-3} per donor respectively. A number of different broad host range vectors have since been conjugated into *Mc.*

capsulatus (Bath) and *Ms. trichosporium* OB3b with transfer frequencies of 10^{-2} to 10^{-8} (Murrell, 1992).

Subsequent work has focused on the heterologous expression of genes in these organisms using two main strategies: (1) expression of sMMO in methanotrophs that naturally possess only pMMO (Martin 1994); (2) complementation of sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b with plasmid-encoded genes (Martin, 1994; Finch, 1997; Chapter 8). The importance of this work has been further increased by the difficulties of expressing active sMMO in a heterologous host (Chapters 3 and 4) and the realisation that this may only be achieved in methane-utilising bacteria.

The first of these strategies relied on the fact that the majority of methanotrophs so far identified only express pMMO and have an obligate requirement for copper (Dalton, 1992) (e.g. *Mm. album* BG8 and *Mcy. parvus* OBBP), whilst a much smaller number possess both pMMO and sMMO (e.g. *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b). Although it is unclear at present why some methanotrophs have two types of MMO, the expression of sMMO may be a survival mechanism in environments where copper limits the growth of methanotrophs capable of expressing only pMMO (Hanson and Hanson, 1996). It has been previously shown that the broad host range vector pVK100 (Knauf and Nester, 1982) was stably maintained in *Ms. trichosporium* OB3b, *Mc. capsulatus* (Bath), *Methylosinus* 6, *Mm. album* BG8 and *Methylocystis* POC (Lidstrom *et al.*, 1984; Murrell, 1992). In addition, pDSK509 (Keen *et al.*, 1988) was stably maintained in *Mc. capsulatus* (Bath) (Murrell, 1992).

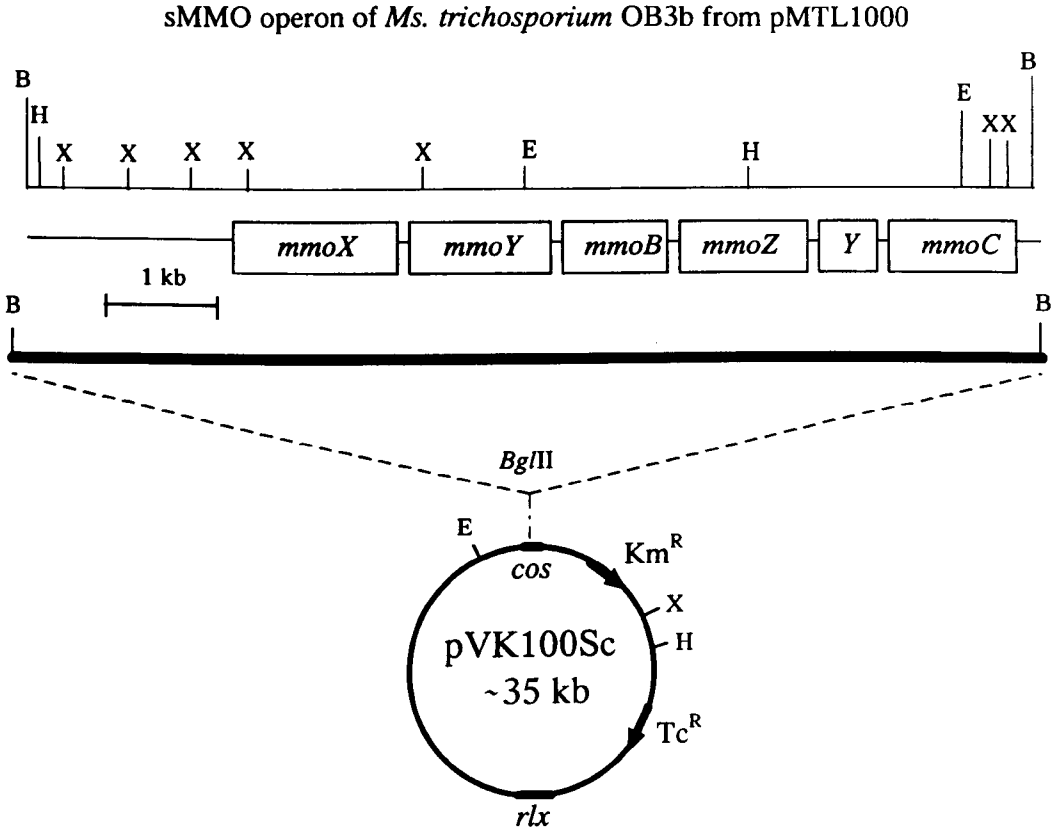
Martin (1994) subcloned the sMMO operons of *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b into pVK100 and pDSK509. It was shown that when pVK104 (pVK100 containing the sMMO operon of *Mc. capsulatus* (Bath)) was conjugated into *Mcy. parvus* OBBP, expression of the reductase component was identified by Western blotting when the transconjugants were cultured in copper-free conditions. Western blotting with anti-sera against the hydroxylase and protein B of *Mc. capsulatus* (Bath) indicated that expression of these polypeptides had not occurred. There have been no studies to determine whether the recombinant reductase expressed in *Mcy. parvus* OBBP [pVK104] was active or whether reductase expression occurred in the presence of copper. Transconjugant strains of *Mm. album* BG8 [pVK104] showed no cross-reactivity with anti-sera against the sMMO components of *Mc. capsulatus* (Bath).

The aim of the work presented in this Chapter was to conjugate broad host range plasmids containing sMMO genes into methanotrophs that only possess gene encoding pMMO. Although the stable maintenance of these plasmids in methanotrophs has been observed, there is only limited evidence to support the heterologous expression of any genes in these organisms.

7.2 Conjugation of broad host range plasmids into methanotrophs

pVK100Sc (Martin, 1994) (Figure 7.1) and pDSK509ΩSc (Martin, 1994) (Figure 8.2) are broad host range plasmids that contain the sMMO operon from *Ms. trichosporium* OB3b. pVK104 (DeMarco, 1996) (Figure 7.2) and pJL-sMMO (Figure 3.8) are broad host range plasmids that contain the sMMO operon from *Mc. capsulatus* (Bath). *Mm. album* BG8 and *Mcy. parvus* OBBP were transformed with these plasmids using the conjugation method described in Section 2.6.3. The

Figure 7.1: Construction of pVK100Sc. A *Bam*HI fragment of approximately 12 kb containing the entire sMMO operon was subcloned into pVK100. Not to scale.



B, *Bam*HI

E, *Eco*RI

H, *Hin*dIII

X, *Xho*I

Y, *orfY*

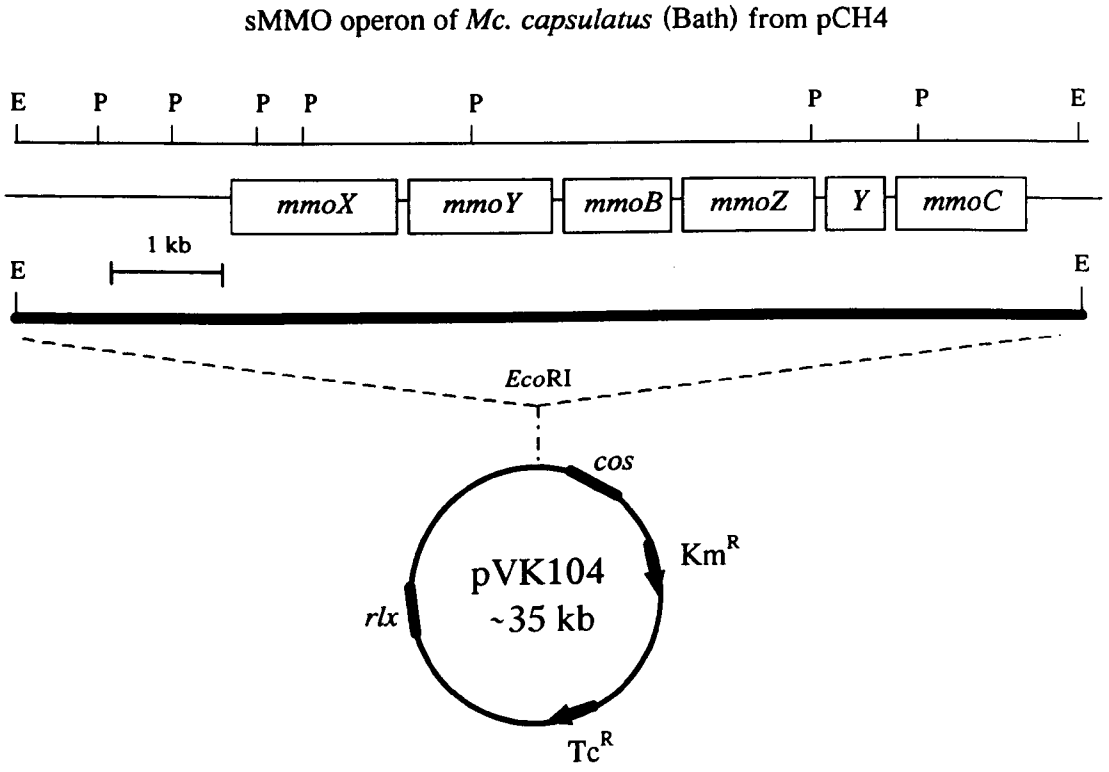
Km^R, kanamycin-resistance gene

Tc^R, tetracycline-resistance gene

rlx, conjugal transfer locus of RK2

cos, cos site of phage λ

Figure 7.2: Construction of pVK104. An *Eco*RI fragment of approximately 12 kb containing the entire sMMO operon of *Mc. capsulatus* (Bath) was subcloned into pVK100. Not to scale.



E, *Eco*RI

P, *Pst*I

Y, *orfY*

Km^R, kanamycin-resistance gene

Tc^R, tetracycline-resistance gene

rlx, conjugal transfer locus of RK2

cos, cos site of phage λ

frequencies of plasmid transfer are described in Table 7.1 and the frequencies of spontaneous antibiotic resistance are shown in Table 7.2.

7.3 Rationale for selection of sMMO expressing transconjugant methanotrophs

To select for transconjugant methanotrophs expressing recombinant sMMO, copper-free growth conditions were used (Section 2.2.3). Evidence suggested that constitutive expression of plasmid-encoded sMMO genes occurred in *Ms. trichosporium* OB3b, even in the presence of copper (Finch, 1997). However, copper-free growth conditions were used to establish if this was also the case for plasmids carrying heterologous sMMO genes in *Mcy. parvus* OBBP and *Mm. album* BG8. Methanotrophs expressing only pMMO will be unable to grow under copper-free conditions due to their obligate requirement for copper. To select for strains capable of growth using pMMO, copper-plus agar plates were used (Section 2.2.3). To screen for the functional expression of sMMO, the naphthalene plate assay was used (Section 2.13.2).

7.4 Heterologous expression of sMMO in *Mm. album* BG8

About 250 colonies of each conjugation of *Mm. album* BG8 [pDSK509ΩSc], [pVK100Sc], [pVK104] and [pJL-sMMO] were replica plated onto copper-plus and copper-free agar, containing the appropriate antibiotics. After three to four weeks growth at 30 °C in the presence of methane, the naphthalene plate assay was performed. Two *Mm. album* BG8 [pVK100Sc] transconjugants were identified that showed a purple coloration on copper-free agar plates only, which was indicative of

Table 7.1: Frequencies of plasmid transfer to *Mm. album* BG8 and *Mcy. parvus* OBBP.

Plasmid	Recipient methanotroph	Antibiotic selection ($\mu g\ ml^{-1}$)[1]	Frequency of transfer per recipient
pDSK509	*Mm. album* BG8	Km (25)	2.8×10^{-6}
	Mcy. parvus OBBP	Km (25)	8×10^{-9}
pDSK509ΩSc	*Mm. album* BG8	Km (25), Sm (10)	3.6×10^{-6}
	Mcy. parvus OBBP	Km (25), Sm (10)	6.1×10^{-8}
pVK100	*Mm. album* BG8	Km (25), Tc (10)	4.2×10^{-7}
	Mcy. parvus OBBP	Km (25), Tc (10)	1.2×10^{-7}
pVK100Sc	*Mm. album* BG8	Km (25), Tc (10)	2.9×10^{-9}
	Mcy. parvus OBBP	Km (25), Tc (10)	3.3×10^{-7}
pVK104	*Mm. album* BG8	Km (25), Tc (10)	7.8×10^{-8}
	Mcy. parvus OBBP	Km (25), Tc (10)	3.5×10^{-9}
pJB3Km1	*Mm. album* BG8	Km (25)	9.2×10^{-9}
	Mcy. parvus OBBP	Km (25)	6.4×10^{-9}
pJL-sMMO	*Mm. album* BG8	Km (25)	5.7×10^{-9}
	Mcy. parvus OBBP	Km (25)	2.9×10^{-9}

[1] Figures in brackets represent the concentration of antibiotic used in $\mu g\ ml^{-1}$.

Table 7.2: Frequency of spontaneous antibiotic resistance in *Mm. album* BG8 and *Mcy. parvus* OBBP.

Methanotroph	Antibiotic selection ($\mu g\ ml^{-1}$)	Frequency of spontaneous antibiotic resistance
Mm. album BG8	Km (25), Tc (10)	$< 10^{-10}$
	Km (25), Sm (10)	$< 10^{-10}$
	Km (25)	$< 10^{-10}$
Mcy. parvus OBBP	Km (25), Tc (10)	$< 10^{-10}$
	Km (25), Sm (10)	$< 10^{-10}$
	Km (25)	5.8×10^{-10}

functional sMMO expression. This suggested that the absence of copper was an important criterion for the functional expression of sMMO in *Mm. album* BG8. The two sMMO-positive transconjugants were designated *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album BG8* [pVK100Sc] 196. A second subculture of *Mm. album* BG8 [pDSK509ΩSc], [pVK104] and [pJL-sMMO] onto copper-free agar followed by the naphthalene plate assay did not identify any further potential sMMO expressing transconjugants. For the remaining 248 *Mm. album* BG8 [pVK100Sc] transconjugants, it was most likely that restriction of the plasmid DNA had occurred such that pVK100Sc was stably maintained but the sMMO genes were restricted or portions of them deleted such that functional expression of the sMMO operon could not be achieved.

7.4.1 Isolation of plasmid DNA

Using a modified alkaline lysis method (Section 2.7.1) plasmid DNA was isolated from *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196. About 20 ng of plasmid DNA was obtained per 50 ml of culture. In order to obtain a restriction map of the plasmid, *E. coli* DH1 was transformed with an aliquot of plasmid DNA and transformants were selected on LB-agar contain Km (25 $\mu g\ ml^{-1}$) and Tc (10 $\mu g\ ml^{-1}$). Isolation and mapping of the plasmid DNA from *E. coli* DH1 transformants confirmed that pVK100Sc was maintained in both transconjugants and was fully intact.

7.4.2 Growth of *Mm. album* BG8 [pVK100Sc] transconjugants on copper-plus and copper-free agar

Mm. album BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 were routinely maintained on copper plus agar containing Km (25 $\mu g\ ml^{-1}$) and Tc (10 $\mu g\ ml^{-1}$). After two or three subcultures of wild-type *Mm. album* BG8 and *Mm. album* BG8 [pVK100] on copper-free agar, biomass yields were four- to five-fold less than those obtained for the same strains on copper-plus agar. Growth on copper-free agar probably occurred due to copper contamination and the ability of *Mm. album* BG8 to scavenge enough copper required for the activity of pMMO. A similar observation was also made by Martin (1994). *Mm. album* BG8 [pVK100Sc] 142 and 196 also gave poor biomass yields on copper-free agar plates in comparison to the biomass yields obtained on copper-plus agar plates after the same length of incubation. After four to five subcultures on copper-free agar, *Mm. album* BG8 [pVK100Sc] 142 and 196 could no longer be subcultured.

To determine if sMMO-specific polypeptides could be identified in *Mm. album* BG8 [pVK100Sc] 142 and 196, whole cells were harvested from copper-free and copper-plus agar plates. Soluble extracts were prepared and separated by SDS-PAGE and then Western blotted with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b (Figure 7.3). Cross-reactivity was observed in *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 grown on copper-free agar plates. The reason for the weaker cross reactivity with the α and γ subunits of the hydroxylase was unclear. Anti-serum against protein B and the reductase from *Ms. trichosporium* OB3b were not available.

7.4.3 Growth of transconjugant methanotrophs in batch culture

Due to the low levels of biomass of *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 obtained on copper-free agar plates, growth of the transconjugant methanotrophs was investigated in liquid culture (Section 2.3.3) (Figure 7.4). In batch grown cultures on copper-plus 1 × NMS, the fastest growth rate determined for wild-type *Mm. album* BG8 was 0.05 h^{-1}, compared to the fastest growth rates of *Mm. album* BG8 [pVK100], *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 which were all about 0.01 h^{-1} under the same growth conditions. Phase-contrast microscopy (Section 2.16.2) of wild-type *Mm. album* BG8 revealed a rod shaped morphology, consistent with the observations of Whittenbury *et al.* (1970). In comparison, *Mm. album* BG8 [pVK100], *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 were mainly cocci shaped. SDS-PAGE profiles of the transconjugant methanotrophs (Figure 7.3) illustrated the high-level of similarity to the wild-type organism, confirming that the

Figure 7.3: SDS-PAGE and Western blotting of sMMO expression in soluble extracts of *Mm. album* BG8 [pVK100Sc] 142 and 196. Lane 1, 10 μg pure hydroxylase from *Ms. trichosporium* OB3b; lane 2, wild-type *Mm. album* BG8; lane 3, *Mm. album* BG8 [pVK100]; lane 4, *Mm. album* BG8 [pVK100Sc] 142; lane 5, *Mm. album* BG8 [pVK100Sc] 196; lanes 6 to 10, Western blot of lanes 1 to 5, probed with anti-serum against the hydroxylase from *Ms. trichosporium* OB3b. All cells were harvested from copper-free agar plates after two subcultures on copper-free agar plates.

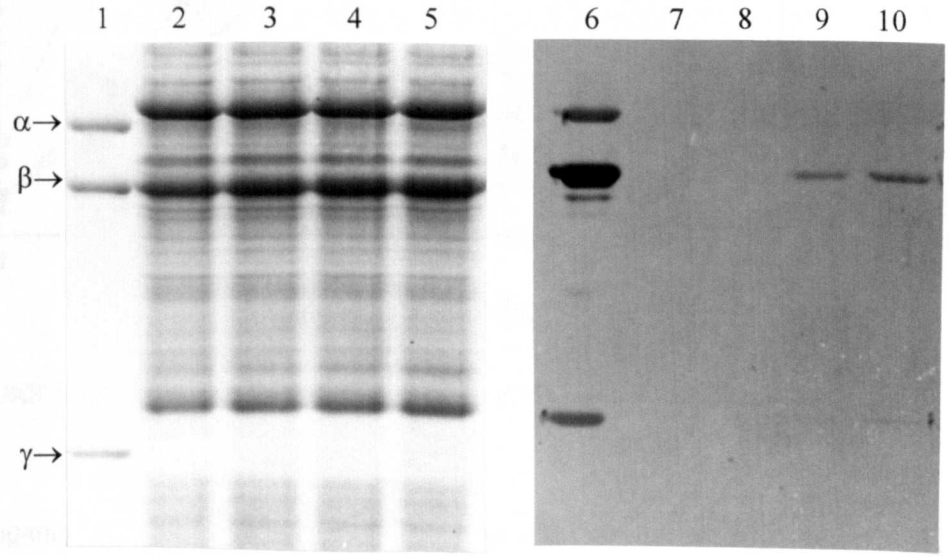
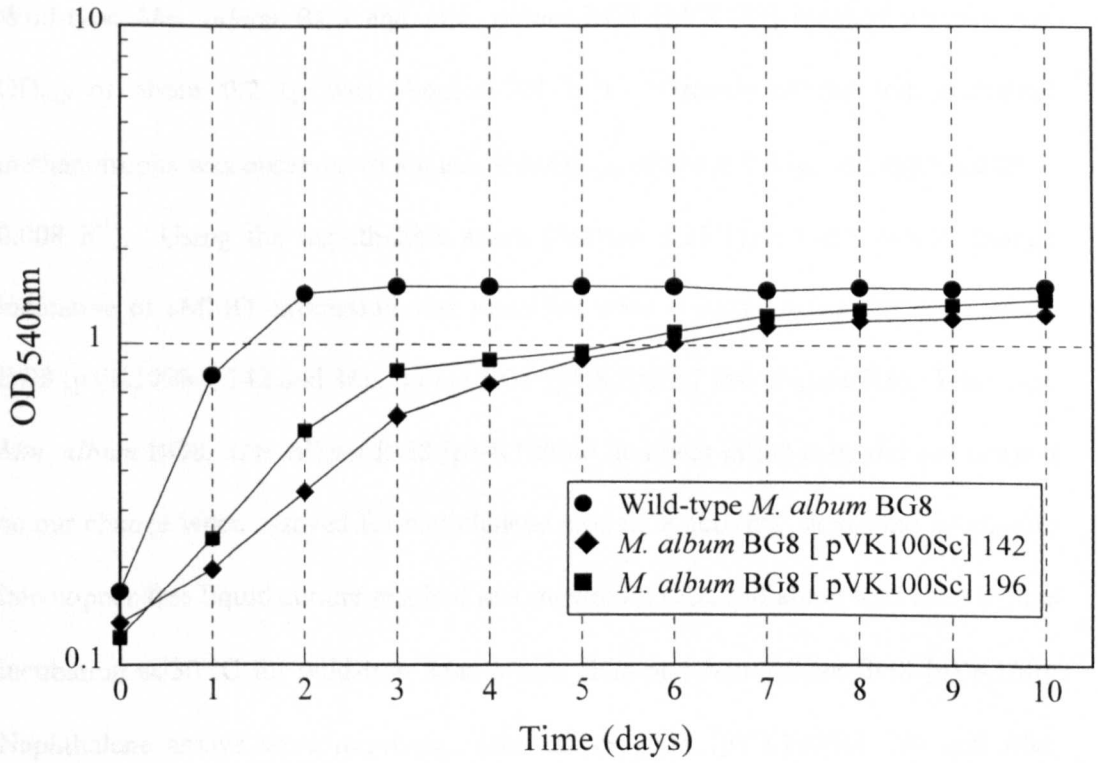


Figure 7.4: Growth of *Mm. album* BG8 [pVK100Sc] transconjugants in copper-plus 1 × NMS. Km (25 μg ml^{-1}) and Tc (10 μg ml^{-1}) were added to 50 ml batch cultures of transconjugant methanotrophs.

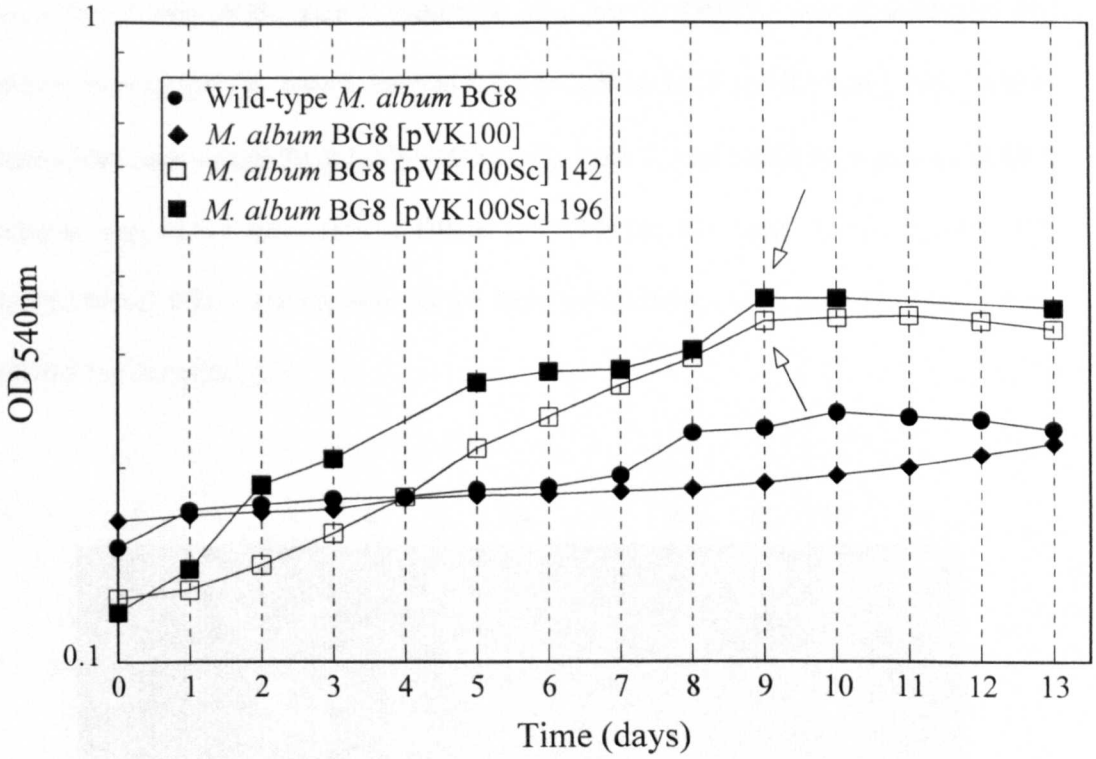


Mm. album BG8 [pVK100] grew at a similar rate to the transconjugant methanotrophs.

transconjugant methanotrophs were unlikely to be contaminating organisms. It was therefore concluded that the antibiotics used for plasmid selection could have caused the change in morphology and possibly caused the reduction in growth rate. Similar observations were made by C. West (University of Warwick, UK). Attempts to optimise the antibiotic concentrations, in order to improve the growth rates of the transconjugant methanotrophs and overcome the change in morphology, proved unsuccessful.

Growth of *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 was investigated in copper-free 0.1 × NMS liquid cultures using *Mm. album* BG8 and *Mm. album* BG8 [pVK100] as negative controls (Figure 7.5). Wild-type *Mm. album* BG8 and *Mm. album* BG8 [pVK100] reached a maximum OD_{540} of about 0.2 (μ was about 0.001 h^{-1}). Growth of the transconjugant methanotrophs was observed to a maximum OD_{540} of about 0.4 (μ was approximately 0.008 h^{-1}). Using the naphthalene assay (Section 2.13.1), a weak colour change indicative of sMMO expression was identified after 9 days growth for *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196 (Figure 7.6). Wild-type *Mm. album* BG8, *Mm. album* BG8 [pVK100Sc] and heat killed cells did not cause a colour change when assayed for naphthalene oxidation activity. A second subculture into copper-free liquid culture resulted in a maximum OD_{540} of about 0.05 after 8 days incubation at 30 °C for wild-type *Mm. album* BG8 and *Mm. album* BG8 [pVK100]. Naphthalene assays were negative. *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142 reached a maximum OD_{540} of about 0.3 after 8 days incubation at 30 °C. Naphthalene assays were positive after 6 days incubation. However, subculturing the methanotrophs for a third time did not result in any growth for *Mm. album* BG8 or *Mm. album* BG8 [pVK100], whilst *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142 reached a negligible maximum OD_{540} of 0.1 after 12 days incubation at 30 °C. Naphthalene assays were negative in all cases. Attempts to grow *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142 in a 2 litre fermenter (Section 2.4.3) using 1 × NMS containing 1 μM copper sulphate were unsuccessful. Three separate attempts resulted in a maximum OD_{540} of 0.3 after 28 days incubation at 30 °C with methane as a sole

Figure 7.5: Growth of *Mm. album* BG8 [pVK100Sc] transconjugants in copper-free 0.1 × NMS. Km (25 μg ml^{-1}) and Tc (10 μg ml^{-1}) were added to 50 ml batch cultures of transconjugant methanotrophs.



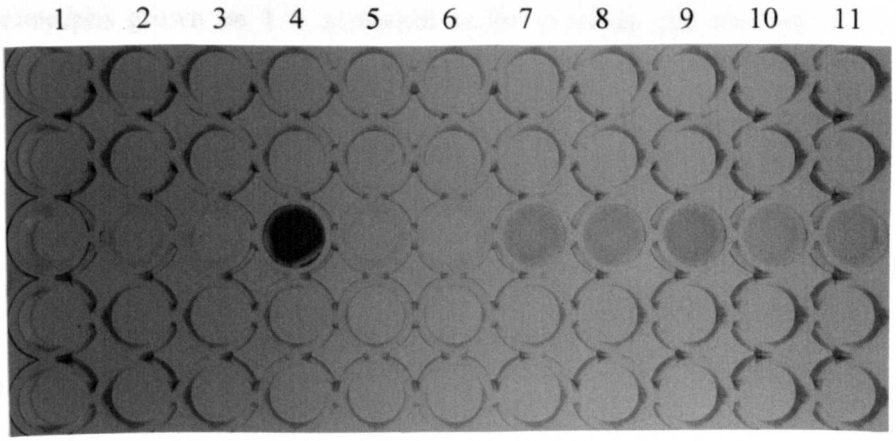
Arrows indicate positive naphthalene assays in *Mm. album* BG8 [pVK100Sc] 142 and *Mm. album* BG8 [pVK100Sc] 196.

carbon and energy source and naphthalene assays were negative.

7.4.4 Growth of transconjugant methanotrophs on methanol

In an attempt to overcome the difficulties encountered with the low growth rates and biomass densities observed with *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142 grown on methane, attempts were made to grow the transconjugant strains on methanol as an alternative carbon and energy source.

Figure 7.6: Naphthalene oxidation assays of *Mm. album* BG8 [pVK100Sc] and *Mcy. parvus* OBBP [pVK100Sc] transconjugant methanotrophs, grown in copper-free 0.1 × NMS. Lane 1, 0.1 × NMS containing Km and Tc; lane 2, wild-type *Mm. album* BG8; lane 3, wild-type *Mcy. parvus* OBBP; lane 4, wild-type *Ms. trichosporium* OB3b; lane 5, heat killed *Mm. album* BG8 [pVK100Sc] 196; lane 6, heat killed *Mm. album* BG8 [pVK100Sc] 142; lane 7, heat killed *Mcy. parvus* OBBP; lane 8, heat killed *Mcy. parvus* OBBP [pVK100Sc] 57; lane 9, *Mm. album* BG8 [pVK100Sc] 196; lane 10, *Mm. album* BG8 [pVK100Sc] 142; lane 11, *Mcy. parvus* OBBP [pVK100Sc] 57.



Utilisation of methanol (*via* methanol dehydrogenase) by the transconjugant methanotrophs in the absence of copper should result in sMMO expression due to the stable maintenance of plasmid-encoded sMMO genes, whilst achieving higher biomass yields. This could potentially overcome poor growth on methane due to low levels of recombinant sMMO expression. In methanol grown *Ms. trichosporium* OB3b, there has been controversy about the expression of sMMO in the absence of copper (discussed in Section 8.8). However, Martin (1994) demonstrated that sMMO expression was identified in methanol grown *Ms. trichosporium* OB3b grown in the absence of copper using the naphthalene assay, but only when cells were washed in 0.1 × NMS prior to assaying. The naphthalene assay results of the transconjugant methanotrophs grown on 1 % methanol in the presence and absence of copper are shown in Table 7.3. sMMO activity was not detected under any of the assay conditions tested.

7.4.5 Propylene oxidation assays of *Mm. album* BG8 transconjugant methanotrophs

Clear difficulties were encountered in the growth of the transconjugant methanotrophs. In an attempt to obtain enough biomass for propylene oxidation assays, 500 ml batch cultures containing copper-free 0.1 × NMS were used. Colonies from copper-plus agar plates were inoculated into shake flasks which were incubated for 9 days at 30 °C in the presence of methane. Soluble and insoluble extracts were prepared as described in Section 2.13.1. The results of the enzyme assays are presented in Table 7.4. Propylene oxidation activity was identified in the soluble extracts of *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142

Table 7.3: Effect of methanol on the growth of transconjugant methanotrophs.

Methanotroph strain	Growth medium (1 % methanol)	OD_{540} after 9 days growth	Naphthalene assay result [1]
Wild-type *Mm. album* BG8	1 × NMS	1.4	Negative
	0.1 × NMS	1.2	Negative
Mm. album BG8 [pVK100Sc] 142	1 × NMS	1.1	Negative
	0.1 × NMS	0.25	Negative
Mm. album BG8 [pVK100Sc] 196	1 × NMS	0.9	Negative
	0.1 × NMS	0.31	Negative

[1] Cells were washed in 0.1 × NMS prior to assaying; 1 × NMS, copper-plus batch culture; 0.1 × NMS, copper-free batch culture. Naphthalene assays after a second subculture did not result in detectable sMMO activity.

only. Western blotting with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b confirmed the expression of the hydroxylase component of sMMO in soluble extracts only.

7.5 Heterologous expression of sMMO in *Mcy. parvus* OBBP

Approximately 250 colonies from each conjugation of *Mcy. parvus* OBBP [pDSK509ΩSc], [pVK100Sc], [pVK104] and [pJL-sMMO] were replica plated onto copper-plus and copper-free agar, containing the appropriate antibiotics. After three weeks growth at 30 °C in the presence of methane, the naphthalene plate assay was performed. No transconjugant strains expressing sMMO could be identified. A control of *Ms. trichosporium* OB3b, grown on the same copper-free medium for the

Table 7.4: Propylene oxidation enzyme assays of wild-type and transconjugant strains of *Mm. album* BG8.

Methanotroph strain	Propylene oxidation activity (nmol min^{-1} mg $protein^{-1}$)
Wild-type *Mm. album* BG8[1]	Insoluble extract: 90 ± 8 Soluble extract: Not detected
Mm. album BG8 [pVK100][1]	Insoluble extract: 56 ± 9 Soluble extract: Not detected
Mm. album BG8 [pVK100Sc] 142	Insoluble extract: 32 ± 6 Soluble extract: 2.5 ± 0.4
Mm. album BG8 [pVK100Sc] 196	Insoluble extract: 26 ± 7 Soluble extract 1.5 ± 0.5

[1] 500 ml batch cultures containing copper-plus 1 × NMS were used. Insoluble extract probably represents pMMO activity and soluble extract probably represents sMMO activity.

same amount of time, gave a positive assay result for sMMO expression. Subsequently, all of the transconjugant methanotrophs were subcultured a second time on to copper-free agar and after two weeks incubation, the naphthalene plate assay was repeated. One *Mcy. parvus* OBBP [pVK100Sc] transconjugant methanotroph was identified that showed a colour change indicative of sMMO activity. This transconjugant, *Mcy. parvus* OBBP [pVK100Sc] 57, was replica plated onto copper-plus and copper-free agar plates and after two weeks incubation, naphthalene assays were again performed. *Mcy. parvus* OBBP [pVK100Sc] 57 brought about a coloration on copper-free agar only, indicative of sMMO expression. To determine if

sMMO expression was responsible for the apparent colour change of the transconjugant methanotroph, whole cells were harvested from the copper-plus and copper-free agar plates for analysis by SDS-PAGE and Western blotting. Expression of the sMMO polypeptides was not visualised by SDS-PAGE stained with Coomassie Brilliant Blue, however, probing with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b confirmed weak cross-reactivity with the α, β and γ subunits of the hydroxylase (data not shown).

In order to obtain enough biomass for propylene oxidation assays, *Mcy. parvus* OBBP [pVK100Sc] 57 was grown in liquid culture. Since wild-type *Mcy. parvus* OBBP contains only pMMO, it should be unable to grow in copper-free 0.1 × NMS batch culture. Martin (1994) showed that this methanotroph was probably a very good scavenger of copper since it required double the number of subcultures on copper-free agar before it could no longer grow, compared to *Mm. album* BG8. To ascertain the number of subcultures that wild-type *Mcy. parvus* OBBP required in copper-free 0.1 × NMS to inhibit growth, 50 ml batch cultures were used. Even after five passages in copper-free 0.1 × NMS, growth of wild-type *Mcy. parvus* OBBP was still evident, albeit it to a lower optical density than that recorded after the first subculture. The same experiments performed with *Mcy. parvus* OBBP [pVK100Sc] 57 did not result in detectable naphthalene oxidation activity even after five passages in copper-free 0.1 × NMS batch culture. In light of this work, *Mcy. parvus* OBBP [pVK100Sc] 57 was grown on a number of copper-free agar plates. After three weeks incubation at 30 °C in the presence of methane, the cells were harvested and an aliquot was resuspended in copper-free 0.1 × NMS for naphthalene oxidation assays. The result of this assay (Figure 7.6) showed a colour change indicative of sMMO

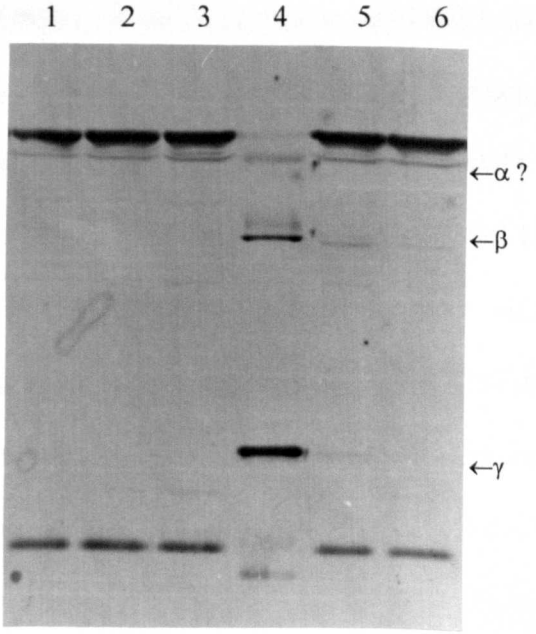
activity. Soluble extracts were subsequently prepared for propylene oxidation assays. Propylene oxidation activity was detected in the insoluble fraction (58 nmol min^{-1} mg $protein^{-1}$), however, no propylene oxidation activity could be detected in the soluble fraction. Western blotting with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b with soluble extract used in the sMMO assays is shown in Figure 7.7.

7.6 Discussion

Heterologous expression of *Ms. trichosporium* OB3b sMMO was demonstrated in *Mm. album* BG8 using pVK100Sc. Although it appeared that *Mcy. parvus* OBBP [pVK100Sc] 57 expressed active recombinant sMMO (as judged by the naphthalene assay), propylene oxidation did not indicate any activity in the soluble extract. The reasons for this are unclear, but may have been due to the instability of recombinant sMMO in soluble extracts, possibly due to the activity of proteases. In contrast to the results obtained in this study, Martin (1994) demonstrated the presence of only *Mc. capsulatus* (Bath) reductase expression in *Mcy. parvus* OBBP.

It is clear from these studies that there are many difficulties in isolating sMMO expressing transconjugant methanotrophs due to the large number of transconjugants which are apparently sMMO-inactive. Since *Mm. album* BG8 and *Mcy. parvus* OBBP both contain plasmids (Lidstrom and Wopat, 1984) then plasmid incompatibility could be a problem. In addition, the large number of antibiotic resistant transconjugants could be explained by restriction barriers in *Mm. album* BG8 and *Mcy. parvus* OBBP such that plasmid-encoded sMMO genes were deleted or restricted. Due to the ability of *Mm. album* BG8 and *Mcy. parvus* OBBP to scavenge

Figure 7.7: Western blot analysis of sMMO expression in soluble extracts of *Mcy. parvus* OBBP [pVK100Sc] 57 probed with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b. Lane 1, soluble extract of *Mcy. parvus* OBBP; lane 2, soluble extract of *Mcy. parvus* OBBP [pVK100]; lane 3, soluble extract of *Mcy. parvus* OBBP [pVK104] 57 grown on copper-plus agar; lane 4, 5 μg pure hydroxylase from *Ms. trichosporium* OB3b; lanes 5 and 6, 40 and 20 μg respectively of soluble extract of *Mcy. parvus* OBBP [pVK104] 57 grown on copper-free agar.



trace amounts of contaminating copper, several copper-free subcultures were required before growth was inhibited. This presents additional difficulties in attempting to select for sMMO expressing transconjugants based upon their ability to grow in copper-free growth conditions.

The effect of copper on the growth of *Mm. album* BG8 has been described by Collins *et al.* (1990). Cultures grown without copper achieved optical densities of 0.075 to 0.175 (as identified in this study), whilst cultures grown in copper supplemented media reached optical densities of 0.65 to 1.05. The obligate copper requirement was 5 to 10 μM copper sulphate and was consistent with the formation of intracytoplasmic membranes. In addition, whole cell MMO activity was increased more than 8-fold when cultures were grown in the presence of copper.

It has been demonstrated that plasmid-encoded sMMO expression in *Mm. album* BG8 [pVK100Sc] 196 and *Mm. album* BG8 [pVK100Sc] 142 was repressed by copper. Nielsen *et al.* (1997) proposed that an active regulator protein was responsible for binding to an upstream activator sequence 5' of the sMMO promoter. The regulator protein makes contact with the RNA polymerase for transcription of the sMMO operon to proceed. Assuming that the proposed model for the copper-dependent regulation of MMO genes was correct, one could postulate that an active regulator protein (or homologue) was also present in wild-type pMMO-only methanotrophs. This could explain the repression of transcription of sMMO genes when grown in the presence of copper, in the sMMO-positive transconjugants. Alternatively, the gene(s) potentially encoding the 'active regulator protein' could have been derived from the 12 kb fragment of DNA present on pVK100Sc, containing the sMMO operon. Analysis of the insoluble extracts of *Mm. album* BG8

[pVK100Sc] 196, *Mm. album* BG8 [pVK100Sc] 142 and *Mcy. parvus* OBBP [pVK100Sc] 57 by SDS-PAGE were performed to establish if constitutive expression of pMMO could be observed. Unfortunately, bands corresponding to the pMMO proteins were not distinguishable and so no conclusions could be drawn. However, constitutive expression of pMMO was observed using the propylene oxidation assay. Although it appeared from this study that the plasmid-encoded sMMO genes were only expressed under copper-free growth conditions, it could be surmised that pMMO expression would be constitutive in wild-type pMMO-only methanotrophs. It would be of little advantage for wild-type *Mm. album* BG8 and *Mcy. parvus* OBBP to repress transcription of the pMMO genes under conditions of copper limitation in the presence of methane, since they do not posses sMMO genes. Assuming wild-type pMMO-only methanotrophs do not have a gene encoding the 'active regulator protein' then heterologous expression of this gene (from pVK100Sc) may not derepress transcription of the pMMO genes in the absence of copper if a different 'active regulator protein' is required to bind to the pMMO operator. It was proposed by Nielsen *et al.* (1997) that different regulator proteins could be responsible for binding to the operator sequences of pMMO and sMMO genes. Subsequently, both pMMO and sMMO could be expressed under conditions of low copper-to-biomass ratios in *Mm. album* BG8 [pVK100Sc] 196, *Mm. album* BG8 [pVK100Sc] 142 and *Mcy. parvus* OBBP [pVK100Sc] 57. Results from the propylene oxidation assays suggest that this was indeed the case. The difficulties in culturing these transconjugant strains may have been due to co-expression of pMMO and sMMO, resulting in a greater metabolic burden on the cell. Concomitant expression of sMMO and pMMO has not been identified in wild-type *Ms. trichosporium* OB3b or *Mc.*

capsulatus (Bath) (Nielsen *et al.*, 1996, 1997). Alternatively, the expression of sMMO or the stable maintenance of pVK100Sc in pMMO-only methanotrophs could be toxic to the heterologous host, resulting in poor growth.

It is unclear why only the sMMO genes of *Ms. trichosporium* OB3b were functionally expressed in pMMO-only methanotrophs. The optimal growth temperature of *Mcy. parvus* OBBP, *Mm. album* BG8 and *Ms. trichosporium* OB3b is 30 °C, in comparison to *Mc. capsulatus* (Bath) which is at 45 °C. The activity of sMMO from *Mc. capsulatus* (Bath) is much lower at 30 °C (Section 4.2.3) and so this could explain the difficulty in isolating transconjugants expressing sMMO from *Mc. capsulatus* (Bath). Also, Nielsen *et al.* (1997) have recently proposed that transcription of the sMMO genes of *Mc. capsulatus* (Bath) are directed from a single σ^{70}-like promoter upstream of *mmoX*. In contrast, expression of sMMO in *Ms. trichosporium* OB3b, is potentially derived from two promoters; a σ^{54}-like promoter directing transcription of the *mmoX* gene and another directing transcription of genes downstream of *mmoX* from a σ^{70}-dependent promoter. It could be postulated that the RNA polymerase of *Mm. album* BG8 and *Mcy. parvus* OBBP only recognised the σ^{54}-like promoter of the sMMO operon of *Ms. trichosporium* OB3b, thus directing the expression of these genes only. Another explanation could be that a molecular chaperone was required for functional expression. Such a gene has been identified 400 bp upstream of *mmoX* of *Ms. trichosporium* OB3b with homology to the GroESL molecular chaperones of *Bacillus stearothermophilus* and *Neisseria gonorrhoeae* (I. R. McDonald and J. C. Murrell, personal communication). The gene encoding this molecular chaperone would be present on the 12 kb insert of *Ms. trichosporium* OB3b in pVK100Sc. An equivalent gene has not been identified in *Mc. capsulatus* (Bath).

The problems encountered with this expression system could be minimised by using broad host range plasmids with strong promoters that could direct the high level expression of sMMO in the transconjugant methanotrophs. In this study, it has been shown that conjugation with *Mcy. parvus* OBBP and *Mm. album* BG8 with the broad host range RK2 plasmid, pJB3Km1 (Blatny *et al.*, 1997) gave rise to antibiotic resistant transconjugants. This suggested that the plasmid was stably maintained. Broad host range RK2 expression vectors have been used successfully for the heterologous expression of the gene encoding phosphoglucomutase (*CelB*) from *Acetobacter xylinum* in *Xanthomonas campestris* (Blatny *et al.*, 1997). If such a system could be used successfully for sMMO then higher expression levels should result in higher enzyme activities for the recombinant sMMO. However, this would not necessarily overcome the poor growth of the transconjugant methanotrophs if the sMMO genes were toxic to the cell, for example.

CHAPTER 8

Complementation of sMMO-minus marker-exchange mutants of *Methylosinus trichosporium* OB3b

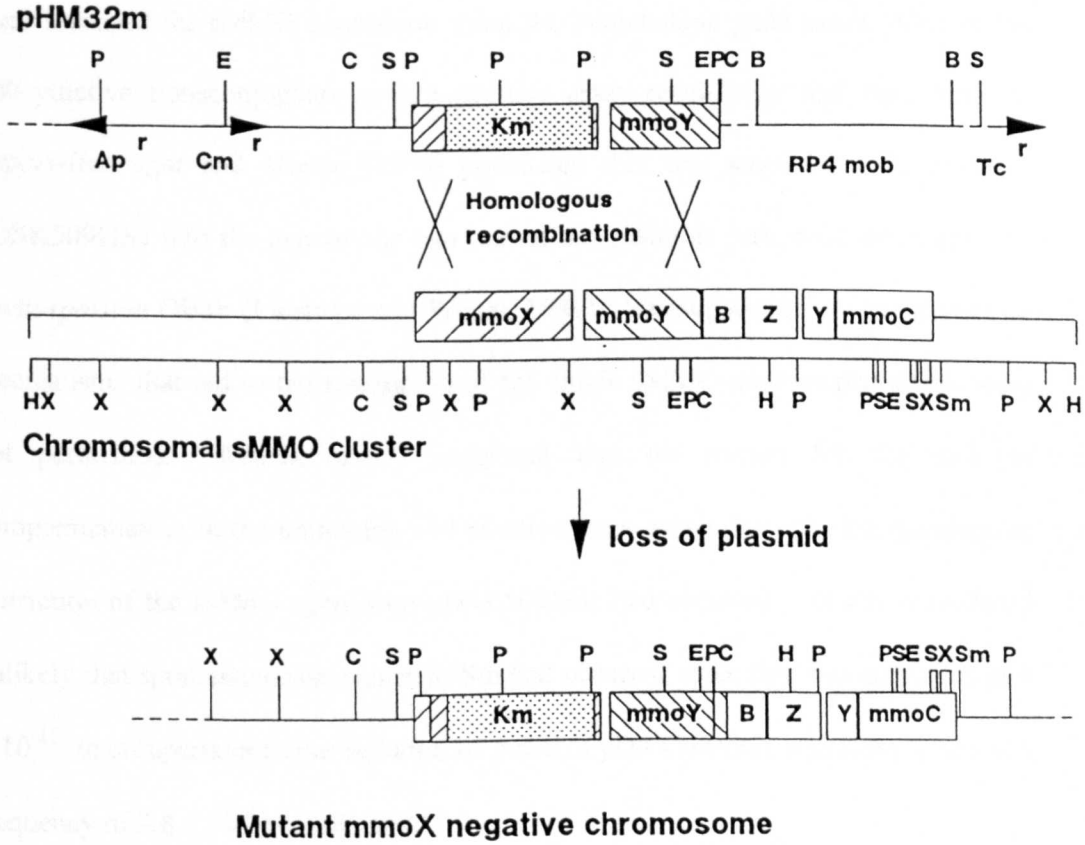
8.1 Introduction

Methanotrophs are difficult organisms to work with in terms of mutant isolation. Mutation frequencies are often low and not increased by the addition of a variety of mutagens, even though the cells are sensitive to such mutagens (Harwood *et al.*, 1972; Martin, 1994). Marker-exchange mutagenesis has been developed as an alternative method for the isolation of mutants inactivated in specific genes. Marker-exchange mutagenesis was first demonstrated in a methanotroph by Toukdarian and Lidstrom (1984) *via* the construction of *nif* mutants of the obligate methanotroph *Methylosinus* sp. strain 6. Marker-exchange mutagenesis normally involves transfer of transposon-containing genes into the recipient cell and the vector is then removed with a conjugative, incompatible plasmid. Due to the problem of a lack of antibiotic resistance markers, a one step marker-exchange procedure was developed that took advantage of the fact that pBR325 was not maintained in *Methylosinus* sp. strain 6. A three-way filter mating procedure was used to mobilise the transposon Tn5 containing *nif* genes cloned into pBR325 into *Methylosinus* sp. strain 6 recipients. After three weeks of incubation at 30 °C with methane, five Km-resistant colonies of *Methylosinus* sp. strain 6 were obtained. Only one *nif*-mutant had arisen from a double-cross-over homologous recombination event, while the four remaining mutants had arisen *via* single-cross-over homologous recombination.

A similar strategy was used to construct stable sMMO-minus mutants of *Ms. trichosporium* OB3b (Martin and Murrell, 1995). This work arose as a means to circumvent the instability of DCM-resistant methane oxidation mutants (Martin, 1994). A 1.2 kb *Xho*I restriction fragment internal to *mmoX* was excised and replaced with a 1.5 kb Km-resistance cassette and then ligated into pBR325. The RP4 specific

mobilisation gene (*mob*) was also ligated into this vector and the construct was designated pHM32m. pHM32m was transferred by filter conjugation into *Ms. trichosporium* OB3b from the donor strain *E. coli* S17-1 (Simon *et al.* 1989) which carries a chromosomal copy of the RP4 transfer functions and so a helper plasmid is not required. Since pBR325 cannot replicate in *Ms. trichosporium* OB3b, Km-resistant colonies should have arisen from homologous recombination between the sMMO-specific DNA carried on pHM32m and the corresponding genes on the chromosome of *Ms. trichosporium* OB3b. This would result in the insertion of a Km-resistance cassette into the wild-type sMMO operon and hence *mmoX*-negative marker-exchange mutants (Figure 8.1). 8 Km-resistant colonies were isolated at a frequency of 5×10^{-8} per recipient. Methanotrophs only capable of growth using pMMO would not be expected to grow on copper-free agar due to the requirement of pMMO for copper. Two of the potential *Ms. trichosporium* OB3b mutants grew as well on copper-free agar as they did on copper-plus agar, suggesting that they had retained the capacity to express sMMO at low copper-to-biomass ratios. The six remaining *Ms. trichosporium* OB3b mutants designated (A) to (F) could not grow in the absence of copper, confirming a sMMO-minus pMMO-positive phenotype. Using the naphthalene plate assay (Brusseau *et al.*, 1990; Graham *et al.*, 1992) (Section 2.13.3) sMMO activity was not detected. Southern hybridisation of the six sMMO-minus mutants confirmed the location of the Km-resistance cassette in the chromosome. Mutants (B) to (F) arose from a double-homologous recombination event since they contained a single modified *mmoX* gene containing the Km-resistance cassette. The chromosome of *Ms. trichosporium* OB3b mutant (A) contained two copies of *mmoX*. This arrangement was probably due to a single

Figure 8.1: Inactivation of *mmoX* by marker-exchange mutagenesis. Double-cross-over homologous recombination would result in the loss of the plasmid and insertion of the Km-resistance cassette into the chromosomal copy of *mmoX*, thereby deleting part of *mmoX*. Figure reproduced from Martin and Murrell (1995).



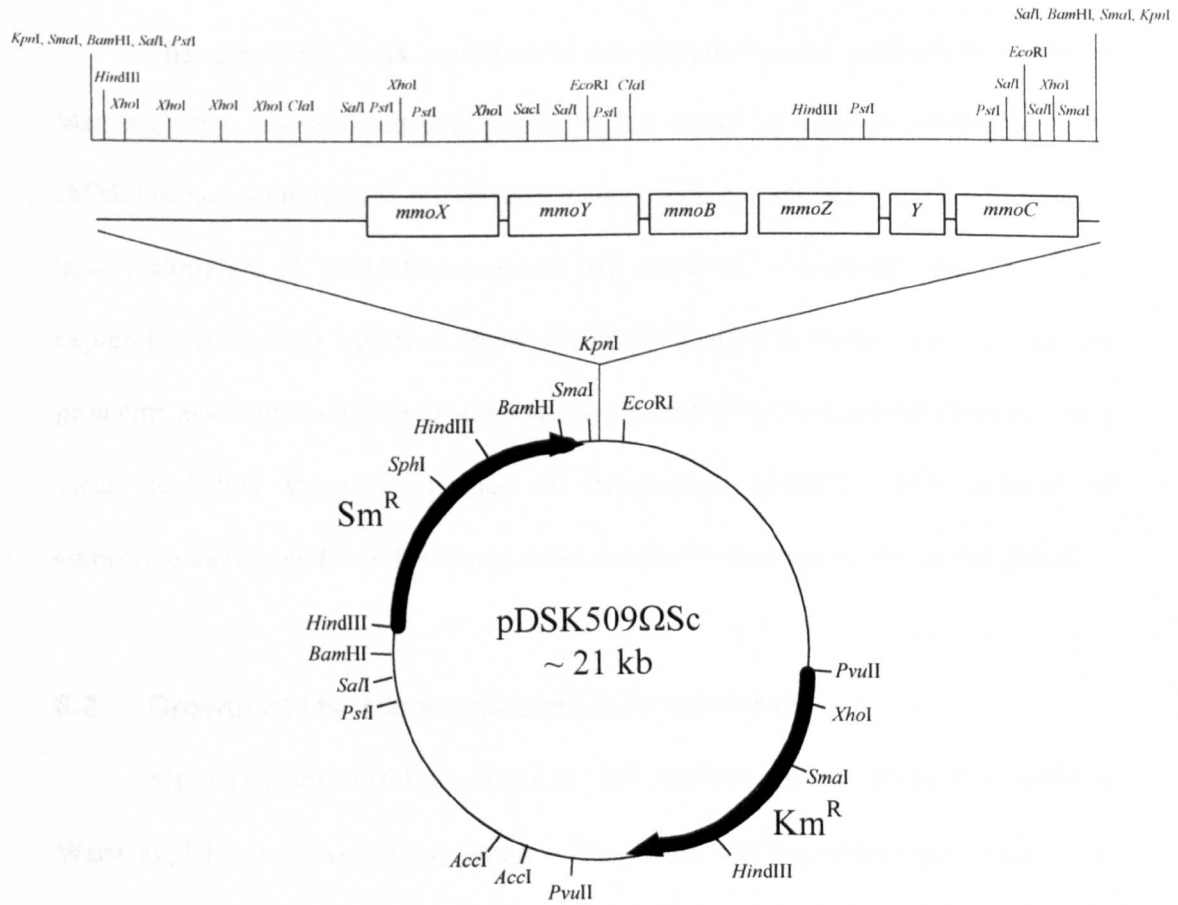
homologous recombination event i.e. pHM32m had integrated into the chromosome.

These two studies conclusively showed that stable methanotroph mutants could be constructed using marker-exchange mutagenesis. However, in both instances careful analysis of the chromosomal DNA of the mutants was necessary to ensure that other recombination events were screened out.

Complementation of sMMO-minus marker-exchange mutants of *Ms. trichosporium* OB3b with plasmid-encoded genes was first demonstrated by Martin (1994). The sMMO-minus phenotype could be restored *via* transformation of *Ms. trichosporium* OB3b mutant (F) with pDSK509ΩSc (Figure 8.2). 200 putative transconjugants were isolated using Sm (20 μg ml^{-1}) for plasmid selection and these were screened for sMMO expression using the naphthalene plate assay. One of the 200 putative transconjugants gave a positive assay result after one subculture on copper-free agar and Martin (1994) postulated that this arose by integration of pDSK509ΩSc into the genome or into one of the plasmids present in wild-type *Ms. trichosporium* OB3b (Lidstrom and Wopat, 1984). Investigations into the molecular mechanisms that led to the restoration of the single sMMO-positive phenotype were not performed. Martin (1994) suggested that the reason for the lack of complementation in the remaining 199 putative transconjugants was that mutation or restriction of the sMMO operon on pDSK509ΩSc had occurred. It was considered unlikely that spontaneous resistance to Sm had occurred since this was estimated at 4×10^{-11}, in comparison to the isolation of putative pDSK509ΩSc transconjugants at a frequency of 2.8×10^{-8} per recipient.

Additional work has involved the complementation of *Ms. trichosporium* OB3b mutant (A) with pVK100Sc (pVK100 containing the sMMO operon from *Ms. trichosporium* OB3b) (Finch, 1997). A single transconjugant was isolated at a frequency of 1×10^{-8} per recipient using Tc (10 μg ml^{-1}) for plasmid selection. Functional expression of sMMO was demonstrated using the naphthalene and propylene oxidation assays. pVK100Sc was retrieved from the transconjugant using a modified alkaline lysis method developed in this study (Section 2.7.1). Inactivation

Figure 8.2: Construction of pDSK509ΩSc. A *Kpn*I fragment containing the sMMO operon of *Ms. trichosporium* OB3b and a *Bam*HI Sm-resistance cassette were ligated into pDSK509 (Keen *et al.*, 1988). Not to scale.



of the chromosomal sMMO operon of *Ms. trichosporium* OB3b mutant (A) with the Km-resistance cassette was confirmed using Southern hybridisation. The results from this work suggested that pVK100Sc was stably maintained in *Ms. trichosporium* OB3b mutant (A) and hence *mmoX* expression was derived from the plasmid-encoded sMMO genes.

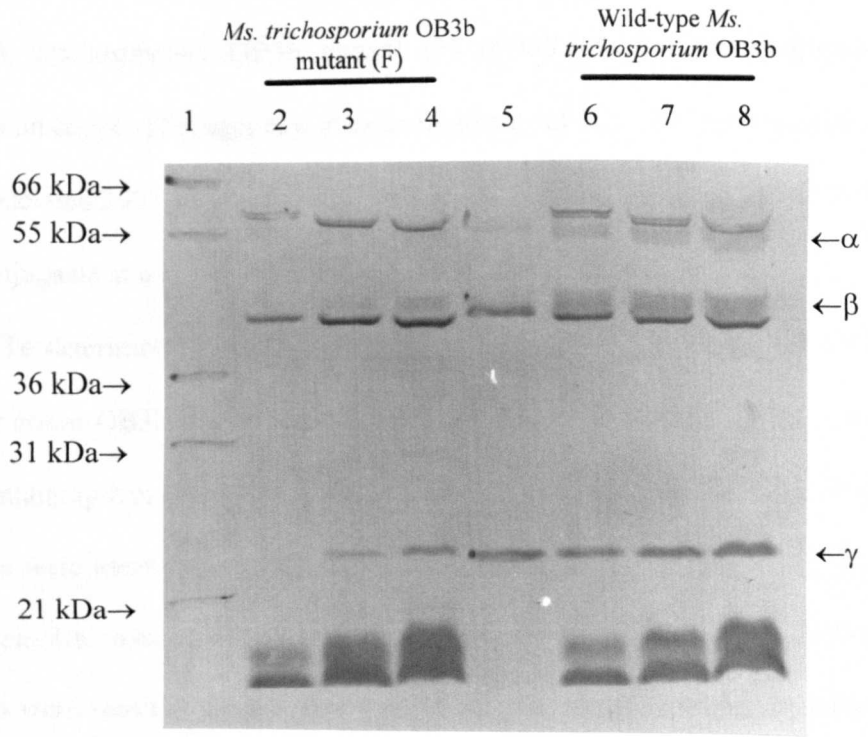
The aim of the work presented in this Chapter was to continue the work of Martin (1994) who showed that pDSK509ΩSc could be used to complement the sMMO-minus phenotype of *Ms. trichosporium* OB3b mutant (F), since it was unclear how restoration of sMMO expression had occurred. Although the functional expression of sMMO has been demonstrated in Chapter 7, there were a number of problems associated with the expression system, including poor growth rates, biomass yields and low enzyme activities of recombinant sMMO. This method of complementation could be developed as an alternative expression system for sMMO.

8.2 Growth of *Ms. trichosporium* OB3b mutant (F)

Ms. trichosporium OB3b mutant (F) was obtained from S. Slade (University of Warwick, UK) as a freeze-dried culture. The culture was inoculated into 50 ml of 1 × NMS (Section 2.3.3) containing Km (25 μg ml^{-1}) and incubated for 7 days at 30 °C using methane as a sole carbon and energy source. Aliquots of the liquid culture were streaked onto copper-plus agar containing Km and incubated for 14 days in the presence of methane at 30 °C (Section 2.4.3). The culture was purified using methods described in Section 2.4.4. To ensure that *Ms. trichosporium* OB3b mutant (F) was unable to express functional sMMO, the naphthalene plate assay was used (Section 2.13.2). 300 single colonies were subcultured onto copper-plus and copper-free agar

(Section 2.4.2) both containing Km (50 μg ml^{-1}). Martin (1994) used Km (25 μg ml^{-1}) to maintain *Ms. trichosporium* OB3b mutant (F) but equally good growth was identified using Km (50 μg ml^{-1}) and so this increased antibiotic concentration was maintained. Wild-type *Ms. trichosporium* OB3b was used as the positive control. After 14 days incubation, naphthalene plate assays confirmed that sMMO expression was not present in any of the *Ms. trichosporium* OB3b mutant (F) colonies grown on copper-plus or copper-free agar. Wild-type *Ms. trichosporium* OB3b gave a positive assay result on copper-free agar only. A second copper-free subculture also resulted in the lack of sMMO expression in *Ms. trichosporium* OB3b mutant (F). Copper-free batch cultures of *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) were grown and after 5 days incubation, naphthalene assays were performed (Section 2.13.2). sMMO expression was detected in the wild-type organism whilst sMMO activity could not be detected in *Ms. trichosporium* OB3b mutant (F) even after 8 h incubation with naphthalene (usual incubation time was 30 min). Growth of *Ms. trichosporium* OB3b mutant (F) probably occurred in copper-free 0.1 × NMS batch culture because trace amounts of copper could have been scavenged for the expression of pMMO. Western blotting with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b was used to investigate the expression of sMMO in these strains (Figure 8.3). In wild-type *Ms. trichosporium* OB3b, cross-reactivity with the α, β and γ subunits of the hydroxylase was seen in comparison to cross-reactivity with the β and γ subunits only, in *Ms. trichosporium* OB3b mutant (F). This was consistent with inactivation of *mmoX* by the insertion of a Km-resistance cassette (Martin and Murrell, 1995).

Figure 8.3: Western blot of wild-type *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) probed with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lanes 2, 3 and 4, 20, 40 and 60 μg respectively of *Ms. trichosporium* OB3b mutant (F) grown in copper-free 0.1 × NMS; lane 5, 5 μg of pure hydroxylase from *Ms. trichosporium* OB3b; lanes 6, 7 and 8, 20, 40 and 60 μg respectively of *Ms. trichosporium* OB3b grown in copper-free 0.1 × NMS.



8.3 Conjugation of pDSK509ΩSc into *Ms. trichosporium* OB3b mutant (F)

Conjugations were performed as described in Section 2.6.3 using pDSK509ΩSc (Martin, 1994) (Figure 8.2) and pDSK509Ω (pDSK509 containing the Sm-resistance cassette) (Martin, 1994) as the negative control. Martin (1994) used Sm (10 μg ml^{-1}) to select for transconjugants but in light of the results obtained in Section 8.2, the antibiotic concentration was increased to Km (50 μg ml^{-1}) (to maintain the selection pressure for the chromosomal and plasmid-encoded Km-resistance cassettes) and Sm (25 μg ml^{-1}) (to maintain selection pressure for the plasmid). 283 *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] transconjugants were selected on copper-plus agar at a transfer frequency of 1.7×10^{-7} per recipient. 312 *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants were selected on copper-plus agar at a transfer frequency of 1.2×10^{-7} per recipient. Martin (1994) isolated 200 Sm-resistant *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants at a transfer frequency of 2.8×10^{-8} per recipient.

To determine the frequency of spontaneous resistance, serial dilutions of *Ms. trichosporium* OB3b mutant (F) were plated onto copper-plus agar and copper-plus agar containing Km (50 μg ml^{-1}) plus Sm (25 μg ml^{-1}) and Sm (25 μg ml^{-1}) only. No colonies were identified on the antibiotic selection plates. Martin (1994) found that wild-type *Ms. trichosporium* OB3b and *mmoX*-minus *Ms. trichosporium* OB3b mutants were sensitive to 5, 10, 20 and 40 μg ml^{-1} of Sm and that spontaneous Sm-resistant mutants were identified only using high cell density cultures from a fermenter at a frequency of only 4×10^{-11} on copper-plus agar containing Sm (20 μg ml^{-1}).

8.4 Identification of sMMO-positive complemented mutants

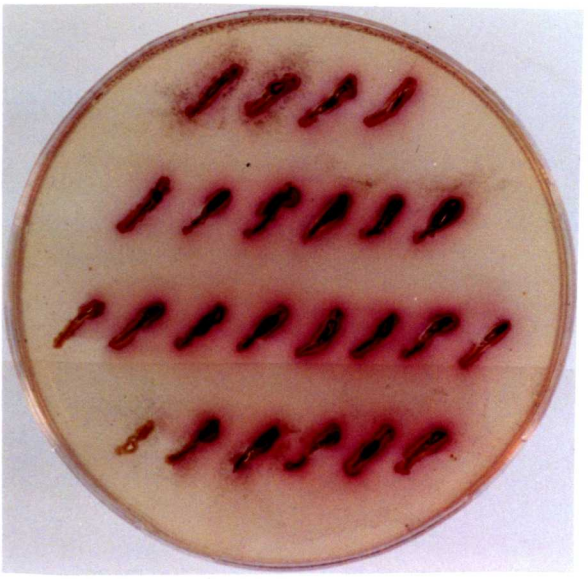
312 *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants were replica plated onto copper-plus and copper-free agar. 283 *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] transconjugants were replica plated in the same manner. After two weeks growth, the naphthalene plate assay was used to screen for those colonies expressing active sMMO. No sMMO-positive *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] transconjugants were identified, however, 3 sMMO-positive *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants designated 6/1, 13/1 and 23/1 were identified (Figure 8.4). The *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants were replica plated onto copper-free agar for a second time and the naphthalene plate assay identified an additional 2 sMMO-positive transconjugants designated 4/2 and 45/2. The five complemented mutants were maintained on copper-free agar containing Km (50 $\mu g\ ml^{-1}$) and Sm (25 $\mu g\ ml^{-1}$).

8.5 Growth of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants in batch culture

The 5 complemented mutants were grown in 50 ml batch cultures of copper-plus 1 × NMS containing Km (50 $\mu g\ ml^{-1}$) and Sm (25 $\mu g\ ml^{-1}$). The growth rates and yields were comparable to those obtained for wild-type *Ms. trichosporium* OB3b. Naphthalene assays were negative for all strains after five days of growth (OD_{540} of 0.6). This was consistent with the copper-to-biomass ratio being high enough to sustain pMMO expression. The organisms were subcultured into copper-free 0.1 × NMS and wild-type *Ms. trichosporium* OB3b was found to express sMMO

Figure 8.4: Naphthalene plate assays of wild-type *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants. A purple/pink coloration is indicative of sMMO expression (Brusseau *et al.*, 1990). (a) wild-type *Ms. trichosporium* OB3b after one subculture on copper-free agar; (b) *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants after one subculture on copper-free agar. Arrows indicate sMMO-positive transconjugants.

(a)



(b)

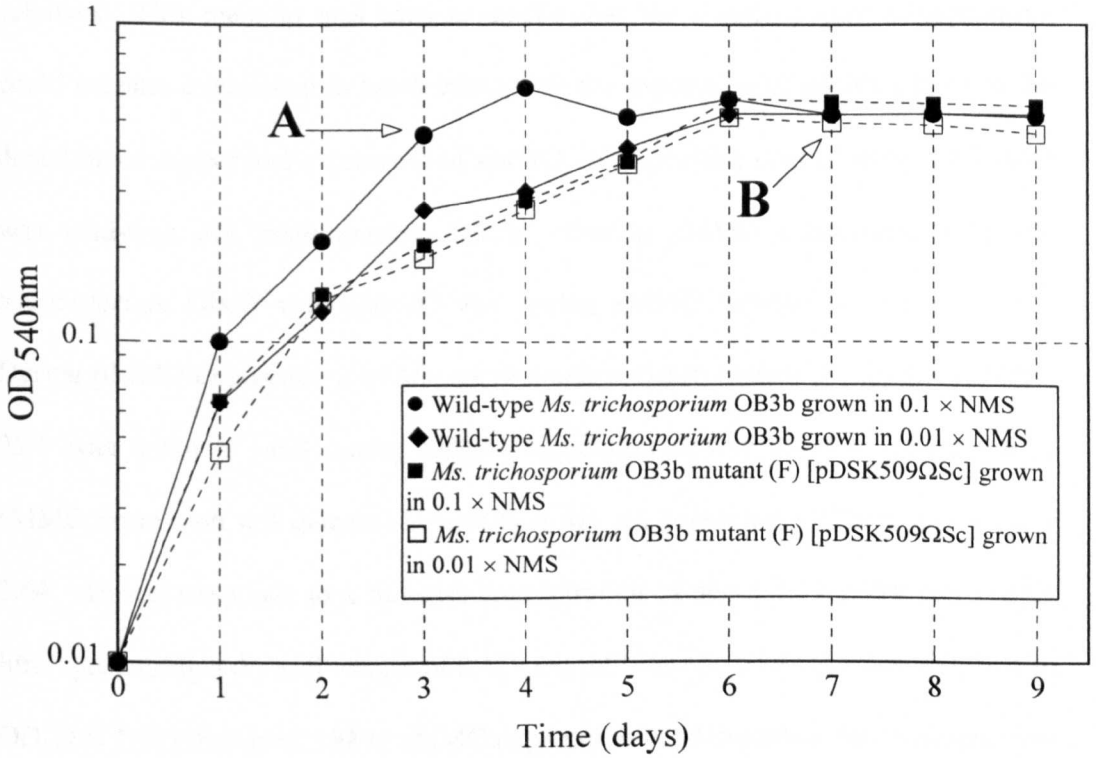
←6/1

←13/1

←23/1

after three days growth (OD_{540} of 0.5) using the naphthalene assay. Naphthalene assays of *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] grown under the same conditions as the wild-type were negative. In addition, naphthalene assays of the five *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants were all negative. Several attempts to achieve sMMO expression in copper-free 0.1 × NMS batch culture for the transconjugant methanotrophs were unsuccessful. To further deplete the amount of copper in batch culture, dilutions of 1 × NMS were made (Figure 8.5). Growth of wild-type *Ms. trichosporium* OB3b in 0.01 × NMS resulted in sMMO expression after 7 days incubation (compared to sMMO expression in 0.1 × NMS, after 3 days incubation). Using the naphthalene assay, sMMO expression was not identified under any of the growth conditions tested for the complemented transconjugant methanotrophs. The reason why sMMO expression was detected on copper-free agar but not in copper-free batch culture was unclear. It was unlikely that the copper-to-biomass ratio was inhibiting the transcription of the sMMO operon (in light of the positive assay results obtained with wild-type *Ms. trichosporium* OB3b grown under the same conditions). Naphthalene assays of the five sMMO-positive *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] transconjugants isolated on copper-free agar after 8 h incubation with naphthalene at 30 °C (usual incubation time for naphthalene assay was 30 min), did result in a weak colour change indicative of sMMO activity. This suggested that a likely explanation for the apparent lack of sMMO activity in batch culture may have been due to a low level of sMMO expression in the transconjugant methanotrophs.

Figure 8.5: Growth of wild-type and transconjugant methanotrophs in 0.1 and 0.01 × NMS.



Data presented for *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] are the mean of the data obtained for all five transconjugants. Point A represents the naphthalene assay detection of sMMO expression in wild-type *Ms. trichosporium* OB3b grown in 0.1 × NMS. Point B represents the naphthalene assay detection of sMMO expression in wild-type *Ms. trichosporium* OB3b grown in 0.01 × NMS.

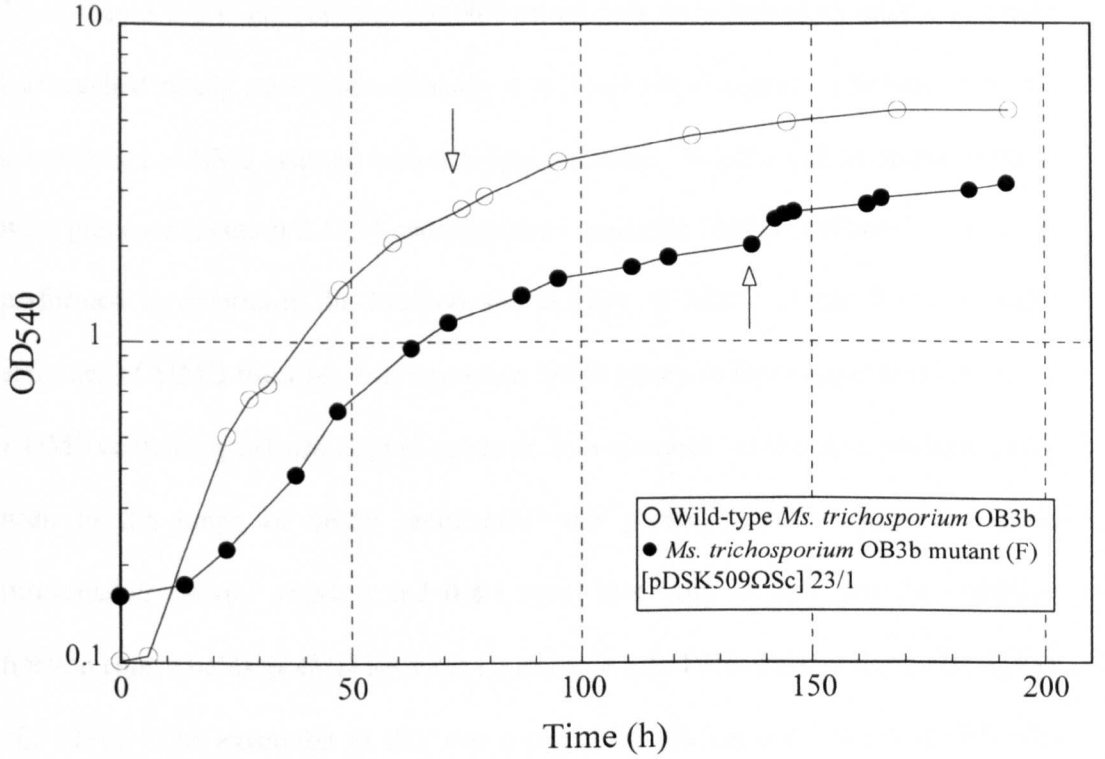
8.6 Batch and continuous culture of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1

8.6.1 Batch culture

In an attempt to increase the weak expression of sMMO in the transconjugant methanotrophs, *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 was grown

in a fermenter so that the copper concentration could be more carefully controlled. A 2 litre fermenter was used (Section 2.4.3) containing 1 × NMS with 1 μM copper sulphate. This medium was used to ensure that the transconjugant methanotroph could become established in batch culture *via* the expression of pMMO, prior to the depletion of copper and expression of sMMO. The positive control used in all cases was wild-type *Ms. trichosporium* OB3b. During pMMO expression, μ of *Ms. trichosporium* OB3b was 0.05 h^{-1} and during sMMO expression was 0.052 h^{-1}. During pMMO expression μ of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 was 0.024 h^{-1} and during sMMO expression it was 0.034 h^{-1} (Figure 8.6). sMMO expression was detected in wild-type *Ms. trichosporium* OB3b at an OD_{540} of 2.64. This corresponds to a biomass concentration of about 1.13 g dry cell weight $litre^{-1}$ presuming a dry cell weight of 0.429 mg ml^{-1} for *Ms. trichosporium* OB3b at an OD_{540} of 1.0 (Pilkington, 1989). sMMO expression was detected in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 at an OD_{540} of 1.85. This corresponded to a biomass concentration of 0.8 g dry cell weight $litre^{-1}$. If these values are presented as the copper-to-biomass ratio then *Ms. trichosporium* OB3b expressed sMMO at a copper-to-biomass ratio of 2.2×10^{-4} g $CuSO_4$ per g^{-1} dry cell weight. *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 expressed sMMO at a copper-to-biomass ratio of 2.0×10^{-4} per g $CuSO_4$ g^{-1} dry cell weight. Burrows *et al.* (1984) reported sMMO activity (detected using the propylene oxidation assay rather than the naphthalene assay) in *Ms. trichosporium* OB3b at 1.7 mg ml^{-1} dry cell weight in 1 μM copper sulphate medium. This corresponds to a copper-to-biomass ratio of 1.5×10^{-4} per g $CuSO_4$ g^{-1} dry cell weight.

Figure 8.6: Growth of *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 in 1 × NMS batch culture containing 1 μM copper sulphate.



Arrows indicate the detection of sMMO activity using the naphthalene assay.

8.6.2 Continuous culture

Finch (1997) showed that *Ms. trichosporium* OB3b mutant (A) [pVK100Sc] constitutively expressed sMMO even in the presence of 3 mg litre^{-1} copper sulphate, which normally allows only pMMO expression in wild-type *Ms. trichosporium* OB3b (Scott *et al.*, 1981; Stanley *et al.*, 1983). To see if this phenomenon was observed in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 the fermenter was switched from batch to continuous culture. The dilution rate in the fermenter was 0.02 h^{-1} and

the OD_{540} of the culture was maintained at 2.2. The control used was wild-type *Ms. trichosporium* OB3b which was grown under identical conditions except that a dilution rate of 0.06 h^{-1} was used to maintain the OD_{540} at 4.0. After the addition of 1, 2 and then 3 mg l^{-1} copper sulphate, 400 ml of cells were harvested once the culture had reached steady state (approximately 4 to 5 volume changes). The basal medium comprised 1 × NMS with no added copper sulphate. Soluble and insoluble extracts were prepared (Section 2.13.1) and propylene oxidation assays (Section 2.13.4) were performed to determine the location and activity of MMO (Table 8.1). Specific activities of MMO from *Ms. trichosporium* OB3b grown in low-copper medium (i.e. 1 × NMS containing 0-1 μM copper sulphate) in continuous culture have predominantly been in the range of 36-80 nmol min^{-1} mg $protein^{-1}$ for the soluble fraction (presumably sMMO activity) and 0-8.4 nmol min^{-1} mg $protein^{-1}$ for the insoluble fraction (presumably pMMO activity) (Burrows *et al.*, 1984; Park *et al.*, 1991; Fox *et al.*, 1991). The exception to this was a report by Stirling and Dalton (1979) who reported MMO activity in the soluble fraction to be 84-146 nmol min^{-1} mg $protein^{-1}$. The MMO activity detected in soluble extracts of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 was comparable to the report of Stirling and Dalton (1979). One hypothesis for the high sMMO activity in the complemented mutant was that there could have been multiple plasmid-encoded copies of the sMMO genes in each cell if pDSK509ΩSc was stably maintained. Alternatively, if pDSK509ΩSc had integrated into the chromosome of *Ms. trichosporium* OB3b mutant (F) by single-cross-over homologous recombination, then duplicate copies of the sMMO operon could be present.

Table 8.1: sMMO to pMMO copper switch in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1.

Copper sulphate concentration (mg litre^{-1})	Specific activity (nmol min^{-1} mg protein^{-1})		
	Wild-type *Ms. trichosporium* OB3b	*Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1	*Ms. trichosporium* OB3b mutant (A) [pVK100Sc][1]
0	Soluble: 42 ± 9 Insoluble: 3 ± 1	Soluble: 140 ± 13 Insoluble: Not detected	Soluble: 54 ± 0.6 Insoluble: Not detected
1	Soluble: 10 ± 4 Insoluble: 18 ± 10	Soluble: 29 ± 3 Insoluble: 21 ± 4	Soluble: 12 ± 0.2 Insoluble: 62 ± 0.3
2	Soluble: Not detected Insoluble: 57 ± 8	Soluble: 4 ± 0.5 Insoluble: 49 ± 9	Soluble: 3 ± 0.4 Insoluble: 65 ± 0.2
3	Soluble: Not detected Insoluble: 63 ± 12	Soluble: Not detected Insoluble: 74 ± 15	Soluble: Not detected[2] Insoluble: 66 ± 0.4

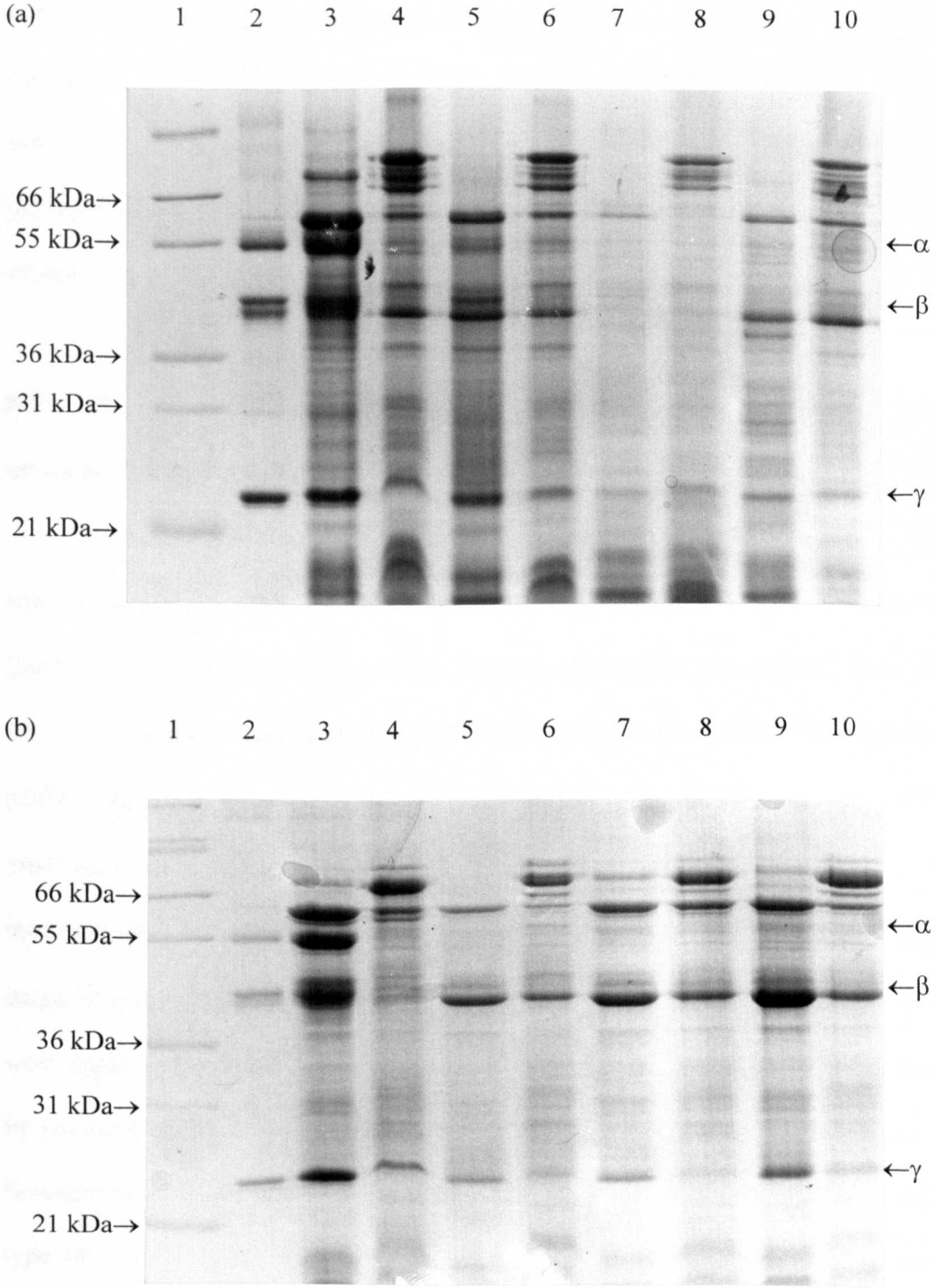
[1] Data obtained from Finch (1997) for comparison; [2] Activity was detected in the soluble fraction by the addition of pure reductase from *Mc. capsulatus* (Bath). Soluble, 1 to 5 mg of soluble extract; Insoluble, 1 mg of insoluble extract. Assays were performed at 30 °C with 481 nmol of propylene and 224 μmol NADH in each assay. The GC retention time of propylene oxide was 2.01-2.05 min in all assays. The results are the mean of triplicate assays.

SDS-PAGE of the *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 copper switch performed in continuous culture is shown in Figure 8.7. Western blotting with anti-serum against the hydroxylase component of *Ms. trichosporium* OB3b (data not shown) confirmed that the hydroxylase component of sMMO was expressed when no copper sulphate was added. At 1-3 mg litre^{-1} copper sulphate added, no cross-reactivity was seen, confirming that repression of transcription of the sMMO operon had occurred. Since propylene oxidation activity was still detected in the soluble fraction at copper sulphate concentrations of 2 mg litre^{-1}, the anti-serum used for Western blotting may not have been sensitive enough to detect the lower concentration of hydroxylase protein. Alternatively, the insoluble fraction containing pMMO may have contaminated the soluble fraction to give rise to propylene oxidation activity. The copper switch results of *Ms. trichosporium* OB3b mutant (A) [pVK100Sc] (Finch, 1997) indicated cross-reactivity with anti-serum against the hydroxylase component of *Ms. trichosporium* OB3b at copper concentrations up to 3 mg litre^{-1}. In contrast to the results obtained with pVK100Sc, constitutive expression of sMMO was not detected using pDSK509ΩSc.

8.7 Isolation of pDSK509ΩSc from *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1

Finch (1997) showed that using the modified alkaline lysis method developed in this study (Section 2.7.1), pVK100Sc could be isolated from *Ms. trichosporium* OB3b mutant (A) [pVK100Sc]. This suggested that functional expression of sMMO was derived from the plasmid-encoded genes. To establish if sMMO expression in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 was due to plasmid-

Figure 8.7: SDS-PAGE of (a) wild-type *Ms. trichosporium* OB3b and (b) *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 grown in continuous culture following the addition of 1 to 3 mg litre^{-1} copper sulphate. Lane 1, molecular mass standards (aprotinin, 6 kDa; lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; lactate dehydrogenase, 36.5 kDa; glutamic dehydrogenase, 55.4 kDa; bovine serum albumin, 66.3 kDa); lane 2, 5 μg of pure *Ms. trichosporium* OB3b hydroxylase; lane 3, 30 μg of soluble extract with no copper sulphate added; lane 4, 30 μg of insoluble extract with no copper sulphate added; lane 5, 30 μg of soluble extract with 1 mg litre^{-1} copper sulphate added; lane 6, 30 μg of insoluble extract with 1 mg litre^{-1} copper sulphate added; lane 7, 30 μg of soluble extract with 2 mg litre^{-1} copper sulphate added; lane 8, 30 μg of insoluble extract with 2 mg litre^{-1} copper sulphate added; lane 9, 30 μg of soluble extract with 3 mg litre^{-1} copper sulphate added; lane 10, 30 μg of insoluble extract with 3 mg litre^{-1} copper sulphate added.


(a)
1 2 3 4 5 6 7 8 9 10
66 kDa→
55 kDa→
36 kDa→
31 kDa→
21 kDa→
←α
←β
←γ
(b)
1 2 3 4 5 6 7 8 9 10
66 kDa→
55 kDa→
36 kDa→
31 kDa→
21 kDa→
←α
←β
←γ

encoded genes, the modified alkaline lysis method was used. *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] was used as the positive control. Plasmid DNA was isolated from *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] which was digested with restriction endonucleases and the restriction pattern was found to be the same as pDSK509Ω from *E. coli* DH1. Plasmid DNA could not be isolated from *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1.

8.8 Southern hybridisation analysis of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1

Integration of pDSK509ΩSc into the chromosome may have been the reason why pDSK509ΩSc could not be isolated from *Ms. trichosporium* OB3b mutant (F) (Section 8.7). To test this hypothesis, chromosomal DNA was isolated from *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 (Section 2.7.5). To ensure that pDSK509ΩSc (21 kb) was not in the single chromosomal DNA band of the CsCl gradient, *E. coli* was transformed with about 200 ng of chromosomal DNA. No transformants were identified on Km (25 μg ml^{-1}) and Sm (10 μg ml^{-1}) agar suggesting that pDSK509ΩSc was not present. Samples of the chromosomal DNA were digested with restriction endonucleases *Pst*I, *Pvu*II, *Sal*I and *Xho*I, fractionated by gel electrophoresis (Section 2.7.7.4) and immobilised onto a nylon membrane *via* Southern blotting (Section 2.8.1). pDSK509ΩSc and chromosomal DNA from wild-type *Ms. trichosporium* OB3b were used as the controls. The Southern blot of these digests was probed with the following radiolabelled fragments (prepared as described in Section 2.8.3): the whole of pDSK509ΩSc; the 1.7 kb *Pvu*II Km-resistance cassette; the 2 kb *Bam*HI Sm-resistance cassette; the 2 kb *Sal*I/*Sac*I fragment of

mmoX. A representative gel of the DNA digests is shown in Figure 8.8. The sizes of chromosomal fragments with homology to pDSK509ΩSc, the Km-resistance cassette, the Sm-resistance cassette and *mmoX* are shown in Tables 8.2 to 8.5. Autoradiographs of the Southern blots are shown in Figures 8.9 to 8.12.

The Km- and Sm-resistant phenotype of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 could have arisen *via* single- or double-cross-over homologous recombination between the chromosomally-encoded sMMO and pDSK509ΩSc, or by complex genetic rearrangements. Results from the Southern hybridisation experiments confirmed that hybridisation was observed between *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 and the Km- and Sm-resistance cassette probes (Tables 8.4 and 8.5). In addition, hybridisation of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 with the whole of pDSK509ΩSc indicated homology with bands of sizes identified in pDSK509ΩSc and wild-type *Ms. trichosporium* OB3b (Table 8.2). However, due to the many different homologous bands that were identified in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1, mapping the sMMO operon proved difficult. This was further complicated by the lack of a detailed restriction map for pDSK509 (Keen *et al.*, 1988). However, the Southern hybridisation results suggested that the Km-resistance cassette in the *mmoX* gene of *Ms. trichosporium* OB3b mutant (F) had been disrupted since the expected homologous band sizes with the Km-resistance cassette and *mmoX* were replaced by different band sizes in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1. This implied that if single-cross-over homologous recombination had occurred then it may have disrupted *mmoX* of *Ms. trichosporium* OB3b mutant (F) (Figure 8.13) and mapping the homologous fragment sizes obtained suggested that this was a possibility

Figure 8.8: Agarose gel electrophoresis of DNA digests. Lanes 1 and 14, 1 kb DNA ladder; lanes 2 to 5, pDSK509ΩSc digested with *Pst*I, *Pvu*II, *Sal*I and *Xho*I respectively; lanes 6 to 9, wild-type *Ms. trichosporium* OB3b chromosomal DNA digested with *Pst*I, *Pvu*II, *Sal*I and *Xho*I respectively; lanes 10 to 13, *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 chromosomal DNA digested with *Pst*I, *Pvu*II, *Sal*I and *Xho*I respectively.

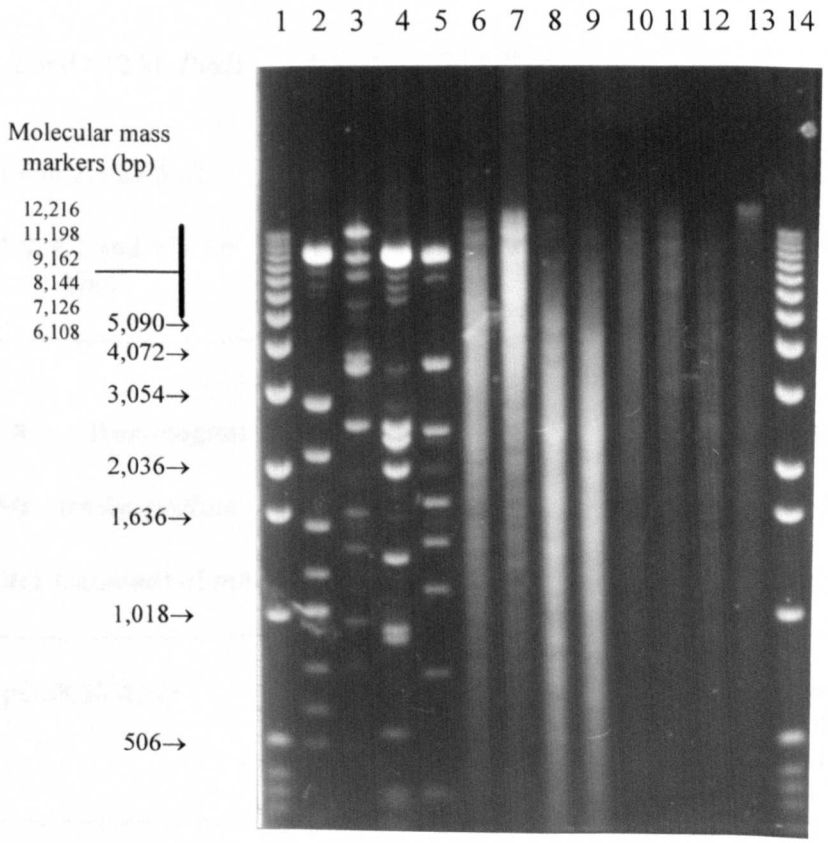


Table 8.2: Homologous band sizes in pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 to the whole of pDSK509ΩSc probe DNA.

pDSK509ΩSc	*Ms. trichosporium* OB3b	*Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1
1.0, 2.2 and 2.9 kb *Pst*I	0.6, 0.9, 1.1 and 2.1 kb *Pst*I	0.6, 0.9, 1.1, 1.8, 2.6 and 3.8 kb *Pst*I
0.9, 4.2 and >12 kb *Pvu*II	0.9 and >12 kb *Pvu*II	2.5, 3.1, 4.0, 6.3 and 12 kb *Pvu*II
2.1 and 2.3 kb *Sal*I	1.3, 1.5 and 2.1 kb *Sal*I	1.3, 2.2, 5.1 and 10 kb *Sal*I
1.2, 1.9, 4.1 and >12 kb *Xho*I	1.1, 1.8 and 3.4 kb *Xho*I	4.2 and 7 kb *Xho*I

Table 8.3: Homologous band sizes in pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 to the 2 kb *Sal*I/*Sac*I fragment of *mmoX* probe DNA.

pDSK509ΩSc	*Ms. trichosporium* OB3b	*Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1
0.5, 1.0 and 2.2 kb *Pst*I	2.2 kb *Pst*I	2.0 and 2.3 kb *Pst*I
0.9, 3.8 and >12 kb *Pvu*II	0.9 and >12 kb *Pvu*II	0.8, 0.9 and 3.6 kb *Pvu*II
2.4 kb *Sal*I	0.6, 1.5 and 2.4 kb *Sal*I	2.3 and 2.6 kb *Sal*I
1.1 and 1.7 kb *Xho*I	1.1 kb *Xho*I	1.1 and 5.5 kb *Xho*I

Table 8.4: Homologous band sizes in pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 to the 1.7 kb *Pvu*II Km-resistance cassette probe DNA.

pDSK509ΩSc	*Ms. trichosporium* OB3b	*Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1
2.8 and >12 kb *Pst*I	NH	1.5, 3.3 and 8 kb *Pst*I
1.2 and 1.5 kb *Pvu*II	NH	1.3, 3.6 and 7.3 kb *Pvu*II
2.8, 3.9 and >12 kb *Sal*I	NH	1.2, 6.3 and 7.2 kb *Sal*I
2.6 kb and >12 kb *Xho*I	NH	4.0 and 9 kb *Xho*I

NH, no hybridisation

Table 8.5: Homologous band sizes in pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 to the 2 kb *Bam*HI Sm-resistance cassette probe DNA.

pDSK509ΩSc	*Ms. trichosporium* OB3b	*Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1
3.2 kb *Pst*I	NH	2.6 and 3.4 kb *Pst*I
5.3, 6.0 and 6.9 kb *Pvu*II	NH	5.1 and 6.6 kb *Pvu*II
2.2 kb *Sal*I	NH	3.9 kb *Sal*I
5.7 kb *Xho*I	NH	3.2 kb *Xho*I

NH, no hybridisation

Figure 8.9: Southern hybridisation of pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 probed with the whole of pDSK509ΩSc. Filter was washed in 0.1 × SSC at 90 °C and exposed for 2 days. Key to lanes is shown in Figure 8.8. Horizontal arrows indicate homologous band sizes to the probe DNA.

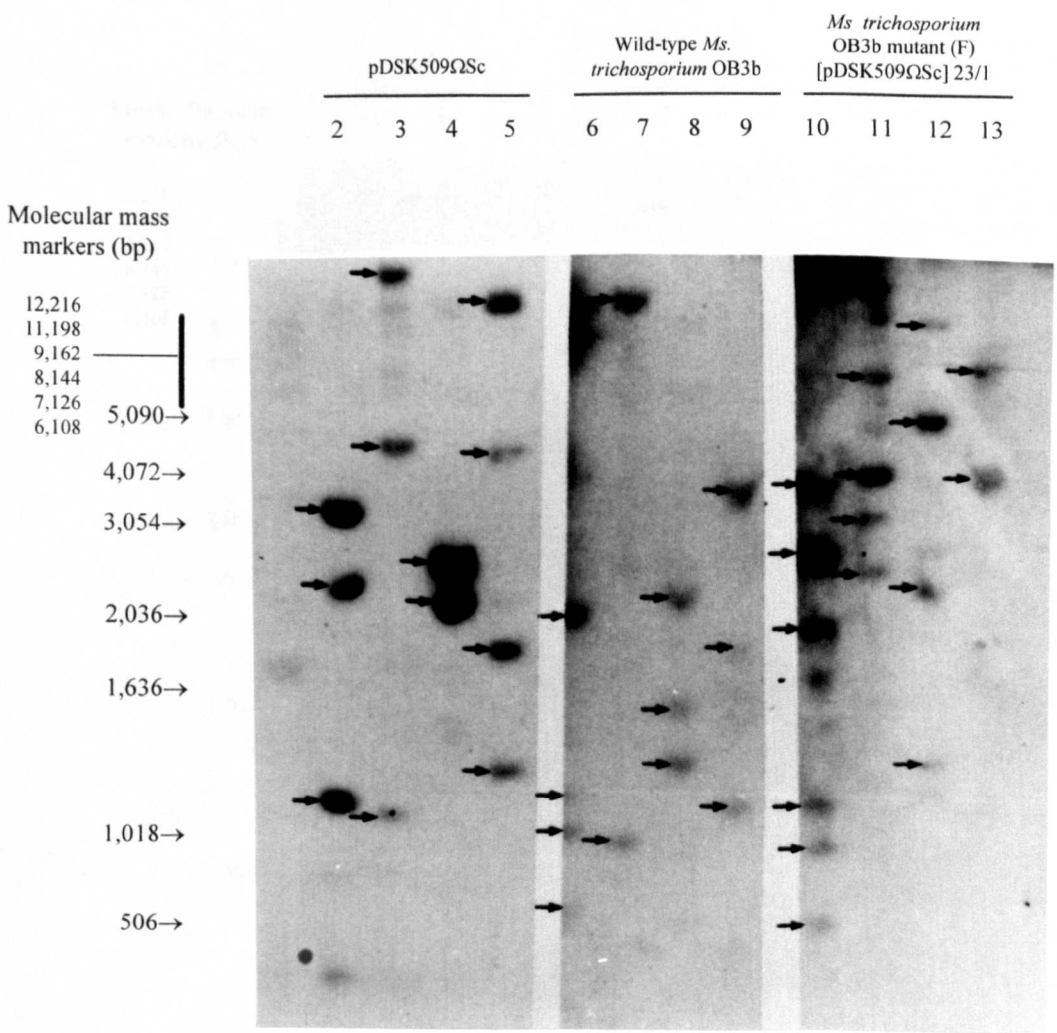


Figure 8.10: Southern hybridisation of pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 probed with the 2 kb *SalI/SacI* fragment of *mmoX*. Filter was washed in 2 × SSC at 65 °C and exposed for 2 days. Key to lanes is shown in Figure 8.8. Horizontal arrows indicate homologous band sizes to the probe DNA.

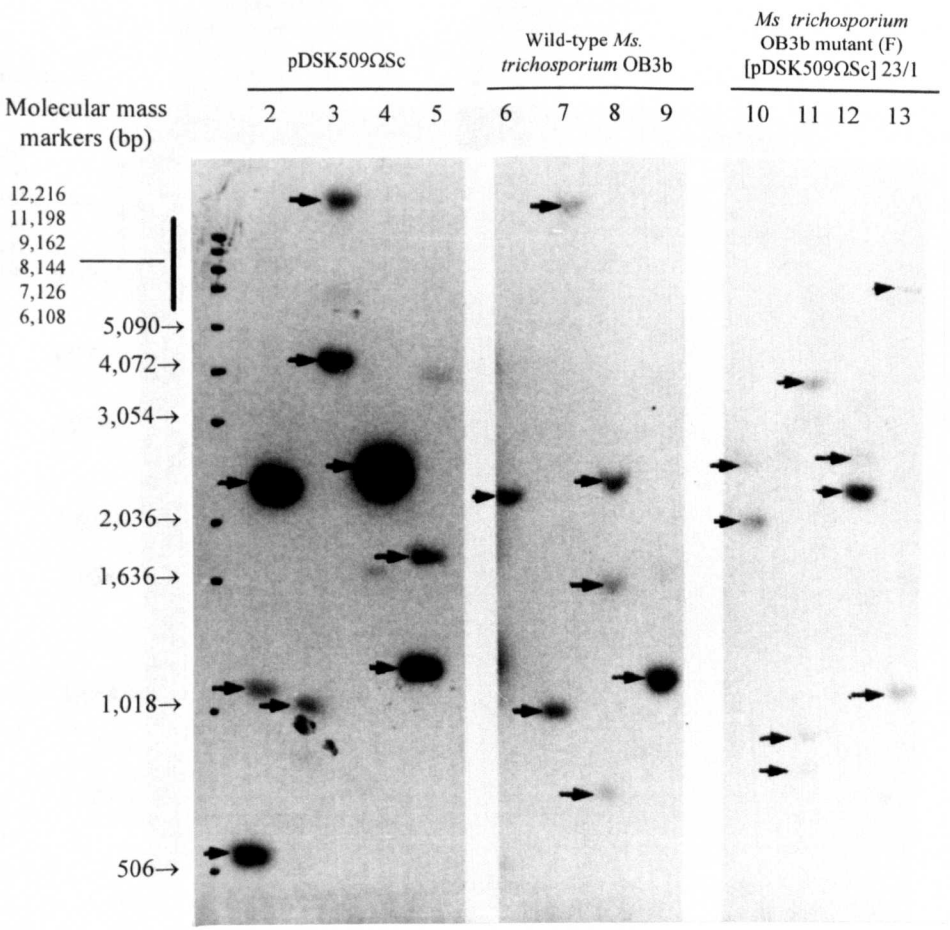


Figure 8.11: Southern hybridisation of pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 probed with the 1.7 kb *Pvu*II Km-resistance cassette. Filter was washed in 2 × SSC at 65 °C and exposed for 2 days. Key to lanes is shown in Figure 8.8. Horizontal arrows indicate homologous band sizes to the probe DNA.

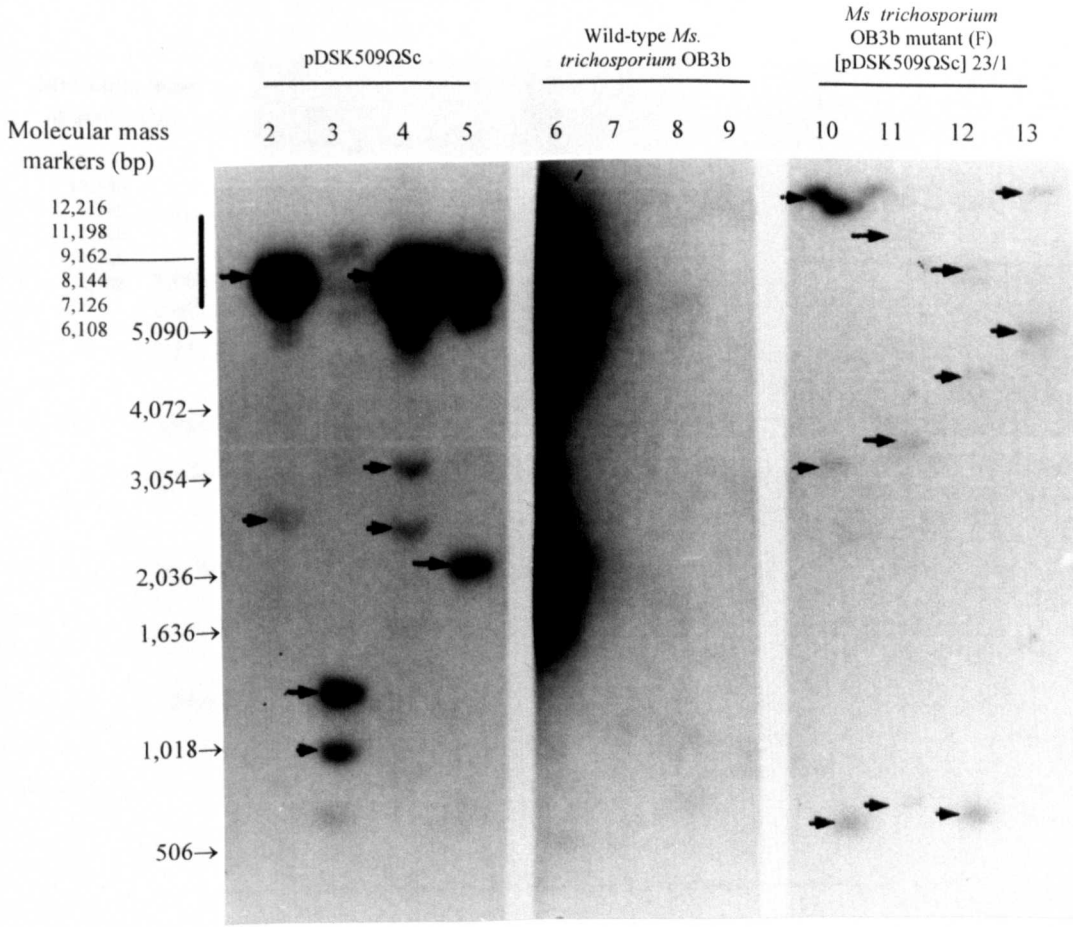
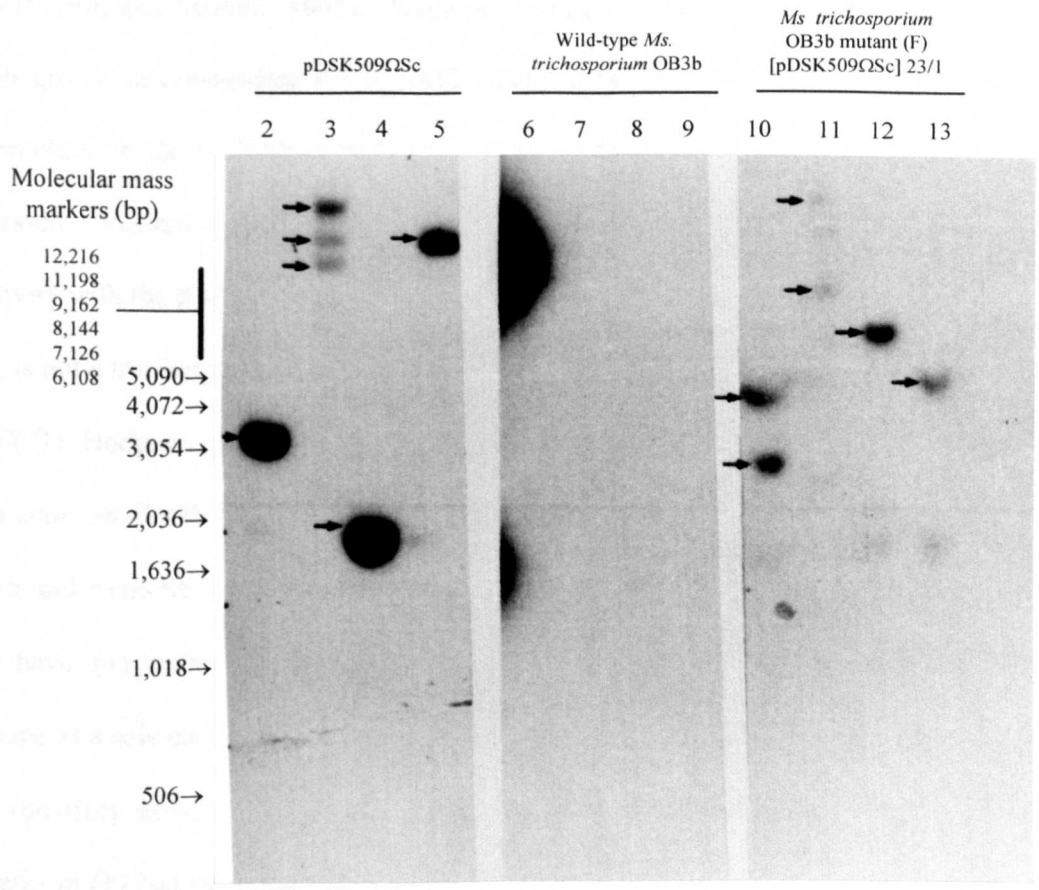


Figure 8.12: Southern hybridisation of pDSK509ΩSc, *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 probed with the 2 kb *Bam*HI Sm-resistance cassette. Filter was washed in 2 × SSC at 65 °C and exposed for 2 days. Key to lanes is shown in Figure 8.8. Horizontal arrows indicate homologous band sizes to the probe DNA.



(Figures 8.14-8.16).

8.9 Discussion

Naphthalene assays of Km-resistant *Ms. trichosporium* OB3b mutant (F) were negative, probably due to the insertion of a Km-resistance cassette into the *mmoX* gene (Martin and Murrell, 1995). Western blotting of wild-type *Ms. trichosporium* OB3b grown in copper-free 0.1 × NMS batch culture with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b confirmed that the α, β and γ subunits were expressed. Western blotting of *Ms. trichosporium* OB3b mutant (F) illustrated cross-reactivity with the β and γ subunits of the hydroxylase which would be expected since there is not a transcriptional terminator within the Km-resistance cassette inserted into *mmoX* (D. Hodgson, personal communication). This observation confirmed that *Ms. trichosporium* OB3b mutant (F) transcribed and translated inactive sMMO when grown under conditions of low copper-to-biomass ratios suggesting that the organism must have: (1) scavenged enough copper to also express pMMO in order to utilise methane as a sole carbon and energy source and therefore grow; (2) entered a dormant state (possibly exospore formation) whilst only inactive sMMO was present. If the scenario in (1) had occurred then there must be a stage of growth during the switch from pMMO to sMMO expression when both forms of MMO are expressed. Nielsen *et al.* (1996, 1997) showed that 10 min after the addition of copper to sMMO-expressing *Mc. capsulatus* (Bath), transcription of sMMO was repressed and pMMO transcription was derepressed. Therefore, it is unlikely that pMMO and sMMO would be concomitantly expressed and so the formation of exospores when only inactive sMMO can be expressed seems the most likely explanation. Martin (1994)

Figure 8.13: Proposed homologous recombination event in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc].

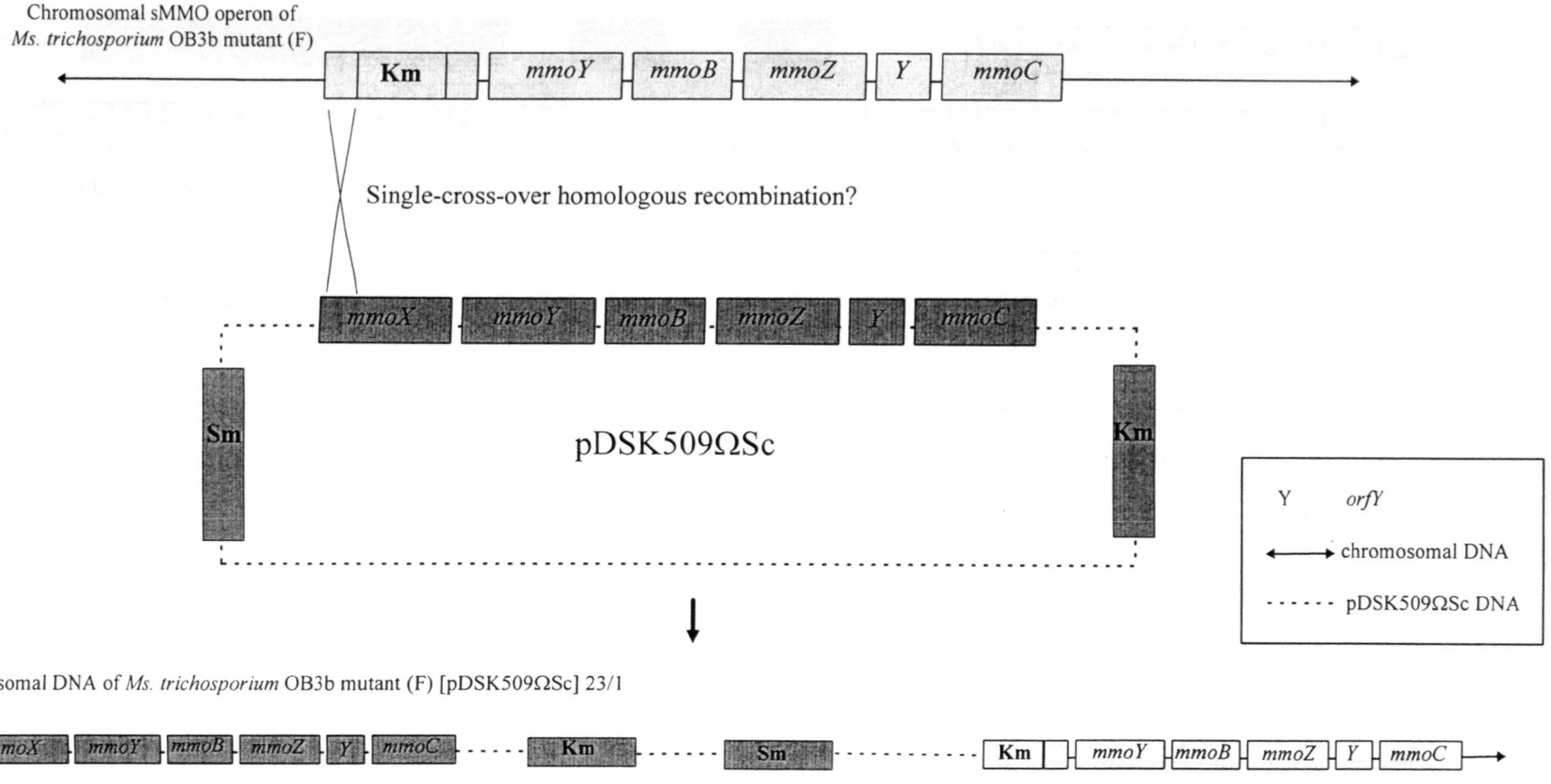
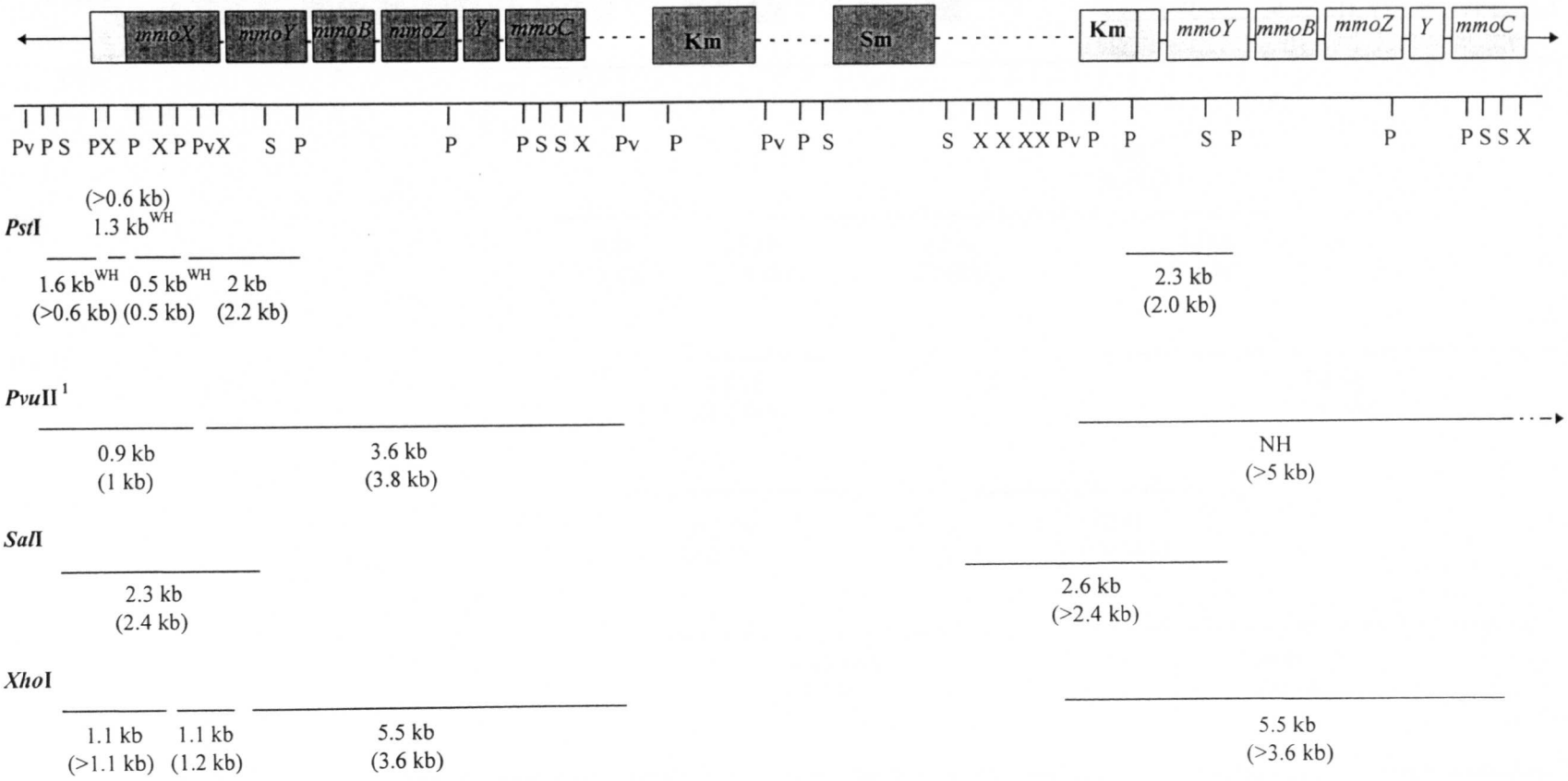
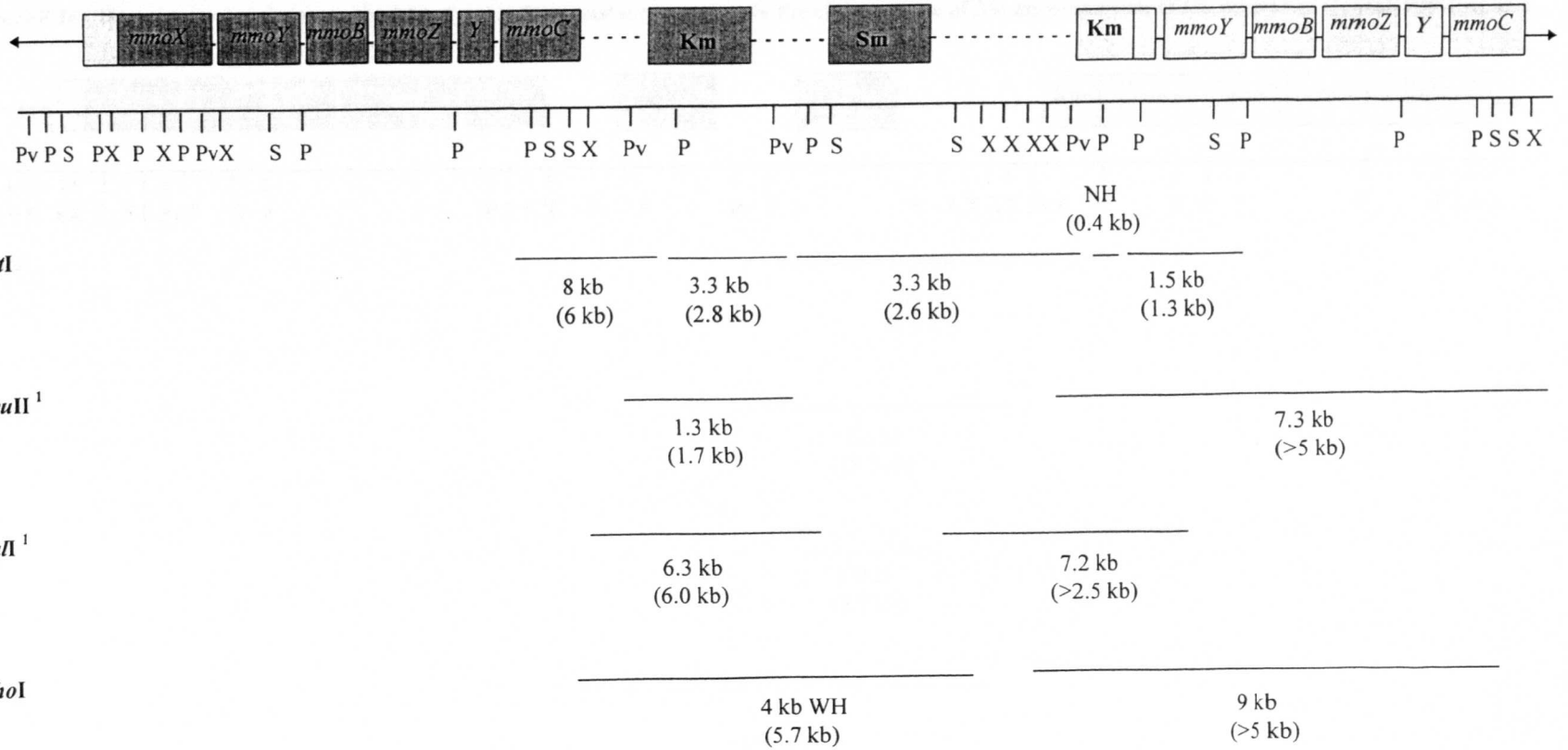


Figure 8.14: Homologous band sizes to the 2 kb *Sal*I/*Sac*I fragment of *mmoX* in the chromosome of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1.



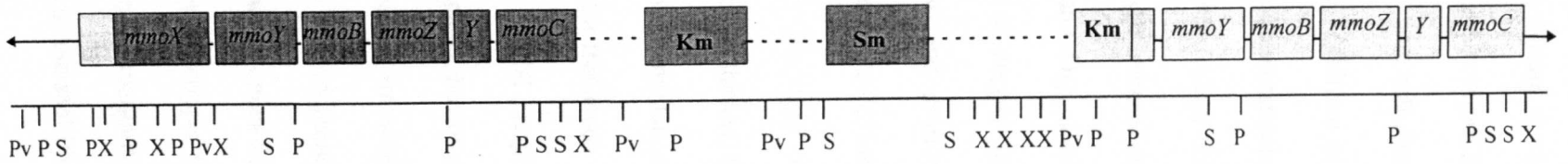
Figures in parenthesis correspond to calculated fragment sizes; [1] Additional homologous bands identified (possibly due to partial digestion); [WH] Weak hybridisation; [NH] No hybridisation. P, *Pst*I; Pv, *Pvu*II; S, *Sal*I, X, *Xho*I.

Figure 8.15: Homologous band sizes to the 1.7 kb *Pvu*II Km-resistance cassette in the chromosome of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1.



Figures in parenthesis correspond to calculated fragment sizes; [1] Additional homologous bands identified (possibly due to partial digestion); [WH] Weak hybridisation; [NH] No hybridisation. P, *Pst*I; Pv, *Pvu*II; S, *Sal*I, X, *Xho*I.

Figure 8.16: Homologous band sizes to the 2 kb *Bam*HI Sm-resistance cassette in the chromosome of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1.



***Pst*I** [1]

2.6 kb
(2.6 kb)

***Pvu*II** [1]

5.1 kb
(5.2 kb)

***Sal*I** [1]

3.9 kb
(2.2 kb)

***Xho*I**

3.2 kb
(<5.7 kb)

Figures in parenthesis correspond to calculated fragment sizes; [1] Additional homologous bands identified (possibly due to partial digestion); [WH] Weak hybridisation; [NH] No hybridisation. P, *Pst*I; Pv, *Pvu*II; S, *Sal*I, X, *Xho*I.

demonstrated that *Ms. trichosporium* OB3b mutant (F) grown in copper-plus 1 × NMS batch culture demonstrated a much slower growth rate than *Ms. trichosporium* OB3b, once the culture had reached an OD_{540} of greater than 7. Consistent with this observation was cell elongation and exospore formation as observed under the light microscope. Addition of 1 mg $litre^{-1}$ copper sulphate to the *Ms. trichosporium* OB3b mutant (F) fermenter caused the dissolved oxygen concentration to decrease by half in 5 min. Within 12 h, the number of elongated cells and spores had also decreased. The wild-type culture grown under the same growth conditions demonstrated sMMO activity at an OD_{540} of about 11. Western blotting of *Ms. trichosporium* OB3b mutant (F) grown in copper-plus continuous culture, probed with anti-serum against the hydroxylase and protein B of *Ms. trichosporium* OB3b showed that no cross-reactivity occurred (Martin, 1994). The reason for the lack of cross-reactivity was most likely the high copper-to-biomass ratio in the growth medium, repressing transcription of the sMMO operon.

Complementation of *Ms. trichosporium* OB3b mutant (F) with pDSK509ΩSc occurred at a frequency of 1.6×10^{-2} in comparison to 5×10^{-3} obtained by Martin (1994). Initial naphthalene plate assays identified three sMMO-positive transconjugants, however, a second copper-free agar subculture identified another two sMMO-positive transconjugants. The remaining 307 sMMO-minus transconjugants may have had an increased sensitivity to copper (thus repressing transcription of sMMO under copper-free growth conditions) or weak sMMO expression. Several subcultures on copper-free agar may have identified a greater number of sMMO-positive transconjugants. Alternatively, Martin (1994) proposed that deletion or restriction of the sMMO operon of pDSK509ΩSc in *Ms. trichosporium* OB3b mutant

(F) could have occurred in order for the *mmoX*-minus complementation to fail. Therefore, pDSK509ΩSc plasmid DNA may not have been susceptible to restriction in the sMMO-positive transconjugants and so *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 may represent a restriction-minus mutant of *Ms. trichosporium* OB3b.

Finch (1997) demonstrated that *Ms. trichosporium* OB3b mutant (A) could be complemented by the stable maintenance of pVK100Sc. Constitutive expression of sMMO was observed under high copper-to-biomass ratios which would normally result in pMMO expression in wild-type *Ms. trichosporium* OB3b (although a wild-type *Ms. trichosporium* OB3b copper switch was not included for comparison). This suggested that the regulation of plasmid-encoded sMMO genes was not under the same regulatory control as the sMMO genes present on the chromosome. Finch (1997) postulated that this was due to multiple copies of plasmid-encoded sMMO genes and hence an increased level of sMMO expression. However, SDS-PAGE of soluble extracts did not indicate a higher level of sMMO expression in *Ms. trichosporium* OB3b mutant (A) [pVK100Sc] in comparison to the wild-type. Also, propylene oxidation assays did not identify higher sMMO activity than in the wild-type. In the present study, one of the complemented mutants, *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 was selected for further analysis. It was shown that the switch from pMMO to sMMO and from sMMO to pMMO expression occurred at the same copper-to-biomass ratios as in wild-type *Ms. trichosporium* OB3b and so the regulation of sMMO expression was under the same control as the wild-type organism. MMO activity in soluble extracts of *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1 grown in copper-free continuous culture were

considerably higher than sMMO activities reported by Finch (1997) and other workers. Only one other report of sMMO activity in *Ms. trichosporium* OB3b by Stirling and Dalton (1979) was comparable to the sMMO activity obtained in this study. Attempts to repeat the work of Finch (1997) using pVK100Sc and *Ms. trichosporium* OB3b mutant (F) failed, due to a high frequency of spontaneous resistance to Tc, which was used for plasmid selection.

Southern blotting demonstrated that pDSK509ΩSc could have integrated into the chromosome by single-cross-over homologous recombination. This highlighted an important comparison between the results obtained in this study with those obtained by Finch (1997). Plasmid DNA was isolated from *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] (this study) and *Ms. trichosporium* OB3b mutant (A) [pVK100Sc] (Finch, 1997) suggesting that these vectors were stably maintained. However, ligation of the sMMO operon from *Ms. trichosporium* OB3b into pDSK509Ω, followed by conjugation into the recipient methanotroph resulted in plasmid integration into the chromosome (at least in the case of *Ms. trichosporium* OB3b mutant (F) [pDSK509Ω] 23/1). It may be that pDSK509ΩSc was stably maintained in the remaining complemented transconjugants but because only one sMMO-positive transconjugant was selected for detailed study then this is not known. In light of the results obtained with the Southern hybridisation experiments, other experiments could be performed to obtain a definitive answer as to the nature of the homologous recombination event in *Ms. trichosporium* OB3b mutant (F) [pDSK509ΩSc] 23/1. PCR could be used to amplify the intergenic regions between the Km-resistance cassettes and both copies of *mmoX* for example. The sizes of the PCR products could then be used to prove or disprove the single-cross-over

homologous recombination hypothesis. In addition, the PCR products could be cloned and sequenced.

Complementation of sMMO-minus mutants of *Ms. trichosporium* OB3b looks set to be the best strategy for expression of sMMO hydroxylase mutants. A major problem in using this method for the expression of mutated (and potentially inactive) hydroxylase is that functional sMMO expression is required for the growth of the organism. If *Ms. trichosporium* OB3b mutant (F) expressed an inactive sMMO then under conditions of low copper-to-biomass ratios the organism could not grow. However, the observation made in this study that *Ms. trichosporium* OB3b mutant (F) will transcribe inactive sMMO genes under conditions of low copper-to-biomass ratios does have major implications for the expression of potentially inactive sMMO genes in this host. An alternative strategy to overcome the problem of inactive sMMO expression would be to grow the organisms under copper limitation with methanol as a sole carbon and energy source. Davis *et al.* (1987) showed that *Ms. trichosporium* OB3b grown in copper-free growth medium with methanol as a sole carbon and energy source expressed sMMO. This was confirmed by Martin (1994) using the naphthalene assay, but only when cells were washed with copper-free 0.1 × NMS. In contrast, Finch (1997) showed that *Ms. trichosporium* OB3b expressed pMMO when grown in copper-free growth medium with methanol as a sole carbon and energy source, an observation also supported by Prior and Dalton (1985) in *Mc. capsulatus* (Bath). This is an area of controversy and further investigations will be required.

The major challenge that will be faced using this expression system will be the identification of transconjugant methanotrophs expressing inactive sMMO. It is

unlikely that all of the hydroxylase mutants will render sMMO inactive, but it is possible that some of the mutations will inactivate the enzyme. Martin (1994) and this study have shown that the frequency of complemented mutant isolation was low representing about 1 % of the total transconjugant population. Fortunately, in this study, the naphthalene plate assay could be used to screen for those transconjugants expressing an active sMMO. One way of identifying cells expressing inactive sMMO would be to prepare whole cell lysates of all the transconjugants grown in copper-free medium and then to probe them with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b. Those transconjugants expressing the α, β and γ subunits of the hydroxylase (in comparison to the β and γ subunits only in *Ms. trichosporium* OB3b mutant (F)) could then be grown in copper-free growth medium with methanol as a sole carbon and energy source and the mutant hydroxylase purified.

CHAPTER 9

Discussion

The choice of host organism can play a crucial role in the heterologous expression of genes. This conclusion can be made after studying the expression of sMMO genes in *E. coli*, *P. putida*, pMMO-only methanotrophs and sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b. Work was initiated with *E. coli* as a host for the heterologous expression of sMMO (West *et al.*, 1992) but inactive hydroxylase was obtained. Using GST-fusion vectors, protein B and OrfY were expressed and purified from *E. coli* by affinity chromatography. This system resulted in the high level expression and simple purification of recombinant proteins, but was limited by the capacity of *E. coli* to express soluble protein. Consequently, the reductase was expressed as insoluble protein, probably due to the inability of *E. coli* to assemble the FAD and Fe_2S_2 centres correctly.

The heterologous expression of proteins in *E. coli* as insoluble inclusion bodies represents one of the biggest problems in using this organism as a heterologous host. Although the renaturation and solubilisation of inclusion bodies can sometimes give rise to active protein, the formation of inclusion bodies is probably a bigger problem than currently appreciated. Many of the expression studies in *E. coli* that result in inactive protein are not published. Certainly, there are systems available that circumvent the formation of inclusion bodies (such as co-expression of thioredoxin for example) but these strategies appear to have an unpredictable effect on the expression of a recombinant protein, sometimes resulting in soluble protein and other times not. This has now led to the general consensus that *E. coli* is a good starting point for the heterologous expression of proteins but is not the definitive answer to the expression of correctly assembled active protein.

P. putida is a simple, wide-spread alternative for the heterologous expression of proteins that are inactive when the genes are expressed in *E. coli*. A good example of the critical choice of heterologous host for active recombinant protein, was the expression of biphenyl dioxygenase (BPH) from *Rhodococcus globerulus* P6, encoded by *bphA* (McKay *et al.*, 1997). No enzyme activity could be detected for BPH expressed in *E. coli* BL21(DE3). To establish why the enzyme was inactive, radioactively labelled transcripts initiated from a T7 promoter *in vivo* indicated that the *bphA* gene products were not present. Gene products downstream of *bphA* were detected suggesting a problem with transcription, translation or degradation. McKay *et al.* (1997) subsequently used the same plasmid construct and BPH activity was detected in *P. putida*. Results published by Jahng and Wood (1994) suggested that a similar scenario was evident for the heterologous expression of sMMO i.e. inactive protein in *E. coli* but active protein in *P. putida*. Attempts to repeat the work gave rise to inactive hydroxylase and a subsequent report by the same group (Jahng *et al.*, 1996) confirmed the results obtained in the present study. There were, however, improvements in the expression of sMMO in *P. putida* compared to *E. coli*, as judged by SDS-PAGE, native-PAGE and Western blotting: (1) SDS-PAGE indicated the expression of the α, β and γ subunits of the hydroxylase in approximate stoichiometric amounts in *P. putida*. In contrast, expression in *E. coli* gave rise to the expression of the β and γ subunits of the hydroxylase but poor expression of the α subunit; (2) sMMO expressed in *P. putida* gave rise to a protein with approximately the same molecular mass as the native hydroxylase from *Ms. trichosporium* OB3b (about 250 kDa), in contrast to *E. coli* where native hydroxylase could not be detected. No enzyme activity was detected for the recombinant hydroxylase expressed in *P. putida*,

suggesting that at least the α subunit was misfolded or the di-iron centre may not have assembled correctly.

Expression of the hydroxylase in *E. coli* and *P. putida* could suggest that an accessory protein may be required for the correct assembly of sMMO. Consequently, the sMMO operon containing *mmoX, mmoY, mmoB, mmoZ, orfY* and *mmoC* may be incomplete and other genes may be involved in the transcription and/or translation of correctly assembled sMMO. A possible candidate for such an accessory protein would be a molecular chaperone: indeed, an open reading frame for a possible molecular chaperone has been identified 400 bp upstream of *mmoX* in *Ms. trichosporium* OB3b. The open reading frame had greatest homology with GroESL of *Bacillus stearothermophilus* and *Neisseria gonorrhoeae* (I. R. McDonald and J. C. Murrell, unpublished). Ellis and deVies (1991) identified the potential problems of heterologous gene expression in terms of the host being unable to assemble proteins into an active conformation, resulting in inclusion body formation. They proposed that the molecular chaperone concept could be an answer and that re-examination of the involvement of molecular chaperones should be undertaken. Ellis and deVies (1991) proposed that if chaperones were found to be involved then the genes should be co-expressed in the same heterologous cells that are expressing the protein of interest in the hope that correct assembly would be favoured. This is a series of experiments that will clearly be required during future research on the heterologous expression of sMMO. A variety of experiments could be performed to assess the role of the putative GroESL homologue during expression of sMMO from *Ms. trichosporium* OB3b. These could include: (1) PCR amplification of the putative *groESL* gene from chromosomal DNA of *Ms. trichosporium* OB3b, followed by

subcloning into a T7-RNA polymerase expression vector. Co-expression of the *Ms. trichosporium* OB3b sMMO operon (from pSMMO20 (Jahng and Wood, 1994) for example) and the putative *groESL* gene from T7 promoters could then be assessed in *E. coli*; (2) marker exchange mutagenesis could be used to integrate an antibiotic resistance gene into the putative chromosomal *groESL* gene of *Ms. trichosporium* OB3b. This would cause inactivation of the gene and hence a lack of putative GroESL expression. If functional expression of sMMO did not occur then this would provide evidence that molecular chaperones were involved in the assembly of sMMO. If functional expression of sMMO was still evident then this could suggest that: (i) molecular chaperones are not involved; (ii) more than one set of genes exist to assist in the correct assembly of sMMO. The identification of a putative GroESL gene upstream of *mmoX* in *Ms. trichosporium* OB3b represents the most exciting prospect to date in achieving functional expression of sMMO in *E. coli* or *P. putida.* This is of such importance due to the simple and rapid genetic techniques that can be used with these organisms. A search for GroESL homologues in other methanotrophs would be worth while.

The heterologous expression of sMMO genes from *Ms. trichosporium* OB3b in pMMO-only methanotrophs and sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b represented the best strategy for the functional expression of sMMO genes in a heterologous host. Although methanotrophs are difficult organisms to manipulate genetically, the use of broad host range plasmids and a reliable transformation system has dramatically improved the situation. Work by Martin (1994) has been especially important during this study, following the use of *E. coli* S17-1 in filter matings to reliably transform plasmids into methanotrophs by

conjugation. In addition, the use of broad host range plasmids has now shown that not only are these vectors stably maintained, but the heterologous DNA is transcribed and translated into active protein, as demonstrated by Finch (1997). Expression of sMMO genes in *Mcy. parvus* OBBP and *Mm. album* BG8 was difficult for a number of reasons: (1) low level of recombinant sMMO expression, resulting in a negligible colour change during the naphthalene assays; (2) growth of wild-type cultures in the absence of copper, making the identification of transconjugants capable of expressing sMMO difficult, in terms of isolation and ensuring that copper levels did not repress transcription of sMMO genes; (3) poor growth of the sMMO expressing transconjugant methanotrophs. However, functional expression of *Ms. trichosporium* OB3b sMMO genes was identified in *Mm. album* BG8, albeit with poor activity in soluble extracts. Optimisation of growth and harvesting conditions may have enhanced recombinant sMMO activity.

Interesting results regarding copper regulation were obtained when the sMMO genes were expressed in *Mm. album* BG8. pMMO expression was constitutive in *Mm. album* BG8 [pVK100Sc] 142 and 196, whilst sMMO expression only occurred in the absence of copper. This suggested that the regulatory elements to repress or derepress pMMO transcription, which must function in *Ms. trichosporium* OB3b and *Mc. capsulatus* (Bath), were not found in *Mm. album* BG8. In contrast, sMMO expression was dependent upon the copper-to-biomass ratio suggesting that the gene(s) encoding the putative activator protein (proposed by Nielsen *et al.*, 1997) was included in pVK100Sc and could have been functionally expressed. Limited success was achieved with *Mcy. parvus* OBBP [pVK100Sc] 57 since propylene oxidation activity could not be detected in soluble extracts. Since positive assay results were

obtained with the naphthalene oxidation assay, then inactivation of sMMO by proteases could have occurred in soluble extracts. Further experiments that include the use of protease inhibitors may result in detectable propylene oxidation activity. In light of the model proposed by Nielsen *et al.* (1997), the results obtained with the sMMO expressing transconjugants provides tentative evidence that the expression of pMMO and sMMO is controlled by different regulator proteins.

The best expression system developed in this study for the future heterologous expression and site-directed mutagenesis of sMMO has been the complementation of sMMO-minus marker exchange mutants of *Ms. trichosporium* OB3b. The construction of sMMO-minus mutants by Martin and Murrell (1995) represented a major breakthrough in the molecular genetics of methane oxidation since the technique of marker exchange mutagenesis represents a powerful tool to knock-out the function of any gene which has been cloned and sequenced. It has overcome previous difficulties in the use of mutagens, including DCM, which have resulted in unstable mutants being isolated at low frequencies. This work was greatly assisted by the use of the naphthalene oxidation assay for sMMO (Brusseau *et al.*, 1990). Attempts to elucidate the molecular mechanism by which sMMO expression was restored in the complemented sMMO-minus mutants obtained in this study was successful, in the sense that homologous DNA fragments to the Sm- and Km-resistance genes were identified. In addition, a model based upon Southern hybridisation results was proposed for the integration of pDSK509ΩSc into the genome of *Ms. trichosporium* OB3b mutant (F) *via* single homologous recombination between the *mmoX* genes. Further elucidation of this model, using PCR for example will be required for a definitive answer. Ultimately, the only way that this system can

be tested to ensure that it has worked successfully is to introduce a mutation into the *mmoX* gene of sMMO *via* site-directed mutagenesis. The vector (engineered in *E. coli*) must then be transformed into *Ms. trichosporium* OB3b mutant (F). Once the complemented mutant has been identified, the recombinant hydroxylase could be purified and the molecular mass of the α subunit determined by ESI-MS. Due to the accuracy of this technique, the theoretical and observed molecular masses could be determined to ensure that the mutated hydroxylase had been expressed and purified.

The clear advantage with this expression system is that the heterologous DNA is under the control of the wild-type *Ms. trichosporium* OB3b promoter. This must be a very strong promoter since expression levels of the hydroxylase can be as high as 60 % of the total cell protein in wild-type *Ms. trichosporium* OB3b. Similar expression levels are therefore achieved with the plasmid-encoded genes integrated into the chromosome. The disadvantage with this expression system for the construction of mutants will be the detection of complemented sMMO-minus mutants, if sMMO is rendered inactive by site-directed mutagenesis. *Ms. trichosporium* OB3b is dependent upon the expression of sMMO to utilise methane as a sole carbon and energy source when grown in the absence of copper. However, expression of sMMO genes was evident in *Ms. trichosporium* OB3b mutant (F) when grown in the absence of copper. The reason for this was unclear because it would be predicted that the organism would switch from pMMO to sMMO expression in the absence of copper. Since the sMMO was inactive (due to the insertion of a Km-resistance cassette), the organism would not express sMMO and hence could not grow. Exospore formation was identified when *Ms. trichosporium* OB3b mutant (F) was grown in the absence of copper (Martin, 1994) and this phenomenon has been studied in detail by Titus *et al.*

(1982). The formation of exospores was induced by maintaining the cells in a methane deficient atmosphere and once formed they were resistant to heat and desiccation and survived passage through a French pressure cell. Therefore, *Ms. trichosporium* OB3b mutant (F) grown in the absence of copper may have a basal expression level of sMMO but enters exospore formation to avoid cell death.

Since the frequency of functional sMMO complemented mutants represented less than 1 % of the total transconjugant population, extensive and time consuming screening will be necessary. The naphthalene assay will not detect complemented mutants if sMMO is inactive and so Western blotting with anti-serum against the hydroxylase of *Ms. trichosporium* OB3b will have to be used to detect for restored expression of *mmoX*. It is unlikely that all of the active site mutations will render the hydroxylase inactive, in which case the naphthalene plate assay could be used to identify the sMMO positive complemented mutants.

Due to time limitations, active site mutations in *mmoX* were not constructed. However, to begin site-directed mutagenesis studies of sMMO, the technique was used with recombinant protein B from *Mc. capsulatus* (Bath). The inactivation of protein B has now been extensively studied by Bhambra (1996) and in this study. It is still unclear whether protein B is inactivated by a specific protease or autocatalysis. However, evidence suggests that it is unlikely to be mediated by a protease since a whole array of techniques designed to minimise proteolytic degradation have been unsuccessful. Of course it is very difficult to prove that a protein is not inactivated by a highly active protease present in only small amounts.

Site-directed mutagenesis of protein B from *Mc. capsulatus* (Bath) successfully improved the stability of purified protein B. Protein B' was identified

after continued incubation at 20 °C, proving that cleavage still occurred at the same cleavage site even after site-directed mutagenesis. This does present difficulties during the crystallisation of protein B because even the mutated protein B is likely to be too unstable to crystallise. However, this cannot be fully judged until crystallisation trials are attempted. Future site-directed mutagenesis experiments to overcome protein B' formation will have to be based upon a random choice of mutation since the only rational decision (based upon the differences in cleavage sites between *Mc. capsulatus* (Bath) and *Ms. trichosporium* OB3b) was used in this study. In addition, the Gln-Val cleavage site that gives rise to protein B'' formation in *Mc. capsulatus* (Bath) is conserved in *Ms. trichosporium* OB3b and *Methylocystis* sp. strain M. Therefore, a rational decision for site-directed mutagenesis cannot be made to overcome protein B'' formation either.

A crystal structure of the hydroxylase bound to protein B would assist in understanding exactly how protein B regulates the activity of the sMMO complex. In *Ms. trichosporium* OB3b this has not been possible (Elango *et al.*, 1997) most likely due to the instability of protein B, as elucidated in this study for the recombinant protein. Purification of protein B from *Ms. trichosporium* OB3b is currently in progress (A. Callaghan and H. Dalton, personal communication) and so it may soon be possible to locate the cleavage site(s) that inactivate protein B from *Ms. trichosporium* OB3b. In addition, extensive *in vivo* studies are currently in progress to determine if protein B' and/or protein B'' can be detected in response to certain environmental conditions, thereby perhaps regulating the activity of the sMMO complex (H. Dalton, personal communication). The same Gln29-Val30 cleavage site has been identified in *Mc. capsulatus* (Bath) and *Mcy.* sp. strain M. If the cleavage

site(s) for protein B are also the same in *Ms. trichosporium* OB3b, then this would further suggest that protein B inactivation acts as a general regulatory mechanism in the methanotrophs. Alternatively, the scissile peptide band could be an incidental consequence of some other essential property of protein B, or a product of protease mediated degradation.

Future work will involve the site-directed mutagenesis of sMMO, which is currently in progress using the expression systems developed in this study (T. J. Smith, J. C. Murrell and H. Dalton, personal communication). The methanotroph-based expression systems are time consuming, but to date have been the only expression systems that have given rise to active recombinant sMMO. It will be important to establish whether the GroESL homologue of *Ms. trichosporium* OB3b is required for functional sMMO expression in *E. coli* and/or *P. putida*, due to the simple genetic systems that have been developed for these organisms.

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APPENDIX

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Inactivation of the regulatory protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) by proteolysis can be overcome by a Gly to Gln modification

John S. LLOYD, Amarbalita BHAMBRA, J. Colin MURRELL and Howard DALTON

Department of Biological Sciences, University of Warwick, Coventry, United Kingdom

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The regulatory protein B of soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), exists as a mixture of the full-length active form and truncated forms, B′ and B″. Electrospray ionisation mass spectrometry (ESI-MS) was used to identify a cleavage site between Met12 and Gly13, such that 12 amino acids were lost from the N-terminus of protein B. This truncate was designated B′ and molecular masses were assigned to proteins B and B′ of 15852.6 ± 0.4 Da and 14629.5 ± 0.3 Da, respectively. A cleavage site between Gln29 and Val30 was also identified such that 29 amino acids were lost from the N-terminus of protein B. This truncate was designated B″ and had a molecular mass of 12709.93 ± 0.02 Da. Proteins B′ and B″ were found to be inactive in the sMMO system. Addition of protease inhibitors or the heterologous expression of protein B in various strains of *lon*-deficient or *ompT*-deficient *Escherichia coli*, did not inhibit B′ formation. Expression of protein B as a glutathione *S*-transferase fusion protein and subsequent purification of protein B from *E. coli* using affinity chromatography resulted in preparations of protein B with higher enzyme activities than that of wild-type protein B. However, ESI-MS confirmed that protein B′ was still present. Alteration of the Met12-Gly13 cleavage site to Met12-Gln13 revealed that the stability of G13Q at 20°C and 37°C was higher than that of wild-type preparations. ESI-MS indicated that protein B′ was absent and could only be identified after prolonged incubation at room temperature. The amount of active protein B present in the cell may be controlled by protein B cleavage, thereby regulating electron transfer. Alternatively, it may allow protein B to maintain a certain conformation necessary for enzyme activity and this may control the activity of sMMO in response to the supply of methane to the cell.

Keywords: methane monooxygenase; site-directed mutagenesis; post-translational modification; proteolytic cleavage; N-terminal processing.

Methanotrophic bacteria can grow on methane as their sole carbon and energy source using the enzyme methane monooxygenase (MMO) to convert methane to methanol in an oxygen-dependent and NADH-dependent reaction. Methanol dehydrogenase then oxidises methanol to formaldehyde, which is assimilated into cellular biomass. Formaldehyde can also be oxidised via formate to carbon dioxide, supplying reducing power for biosynthesis and methane oxidation. All methanotrophs express a membrane bound particulate methane monooxygenase (pMMO) (Zahn and DiSpirito, 1996) although some also express a cytoplasmic soluble methane monooxygenase (sMMO) (Dalton et al., 1993; Lipscomb, 1994). This differential expression is dependent upon copper ions in the growth medium; a high copper/biomass ratio results in pMMO expression and a low copper/biomass causes expression of sMMO (Stanley et al., 1983; Nielsen et al., 1996).

sMMO has been purified and characterised from a number of organisms including *Methylococcus capsulatus* (Bath) (Colby and Dalton, 1978), *Methylosinus trichosporium* OB3b (Fox et al., 1989) and *Methylocystis* sp. M (Nakajima et al., 1992). Three protein components, the hydroxylase (protein A), the reductase (protein C) and a regulatory protein B are required for activity of sMMO.

In *M. capsulatus* (Bath) the hydroxylase (250 kDa) is comprised of two copies of each of the three subunits, α (60 kDa), β (45 kDa) and γ (20 kDa) (Woodland and Dalton, 1989; Dalton et al., 1993). It contains a bridged diiron centre within an active site, where methane and dioxygen interact to form methanol (George et al., 1996). X-ray crystal structures of the hydroxylase from *M. capsulatus* (Bath) (Rosenzweig et al., 1993) and *M. trichosporium* OB3b (Elango et al., 1997) have been reported. The reductase (38.5 kDa) is a single polypeptide and contains an FAD group and one Fe_2S_2 centre per molecule. It accepts electrons from $NADH_2$ and transfers them one at a time to the diiron site of the hydroxylase (Lund and Dalton, 1985; Lund et al., 1985). Protein B (16 kDa) is a single subunit protein containing no metals, prosthetic groups or cofactors (Green and Dalton, 1985; Liu and Lippard, 1991). Occasionally, protein B has been observed to have a molecular mass of 31 kDa by gel filtration,

Correspondence to H. Dalton, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK

Fax: +44 1203 523568.

E-mail: HD@dna.bio.warwick.ac.uk

URL: http://www.oikos.warwick.ac.uk

Abbreviations. ESI-MS, electrospray ionisation mass spectrometry; FTICR-MS, Fourier transform ion-cyclotron resonance mass spectrometry; GST, glutathione *S*-transferase; MMO, methane monooxygenase; pMMO, particulate methane monooxygenase; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; sMMO, soluble methane monooxygenase.

Enzyme. Methane monooxygenase (EC 1.14.13.25).

suggesting that it is capable of existing as a dimer in *M. trichosporium* OB3b (Fox et al., 1989). A similar observation has also been made in our laboratory for the *M. capsulatus* (Bath) enzyme. Recent reports indicate that protein B from *Methylocystis* sp. M also exists as a dimer consisting of two identical subunits (Uchiyama, H., unpublished results). Protein B has been suggested to have a number of roles including: increasing rates of reactions by 150-fold (Fox et al., 1991); changing the spectroscopic properties of the hydroxylase (Fox et al., 1991); shifting the redox potential properties of the hydroxylase (Paulsen et al., 1994; Kazlauskaite et al., 1996); altering the product distribution for complex substrates that can be hydroxylated in more than one position (Froland et al., 1992); varying the rate of product formation when the hydroxylase functions through a H_2O_2 shunt (Froland et al., 1992); increasing the rate of electron transfer, coupling substrate oxidation to NADH consumption (Green and Dalton, 1985; Liu and Lippard, 1991); altering the structure of the hydroxylase diiron cluster (Davydov et al., 1997).

Although protein B from *M. trichosporium* OB3b has a 89.4% amino acid sequence similarity and 66% amino acid identity to sMMO from *M. capsulatus* (Bath) (Cardy et al., 1991; Murrell, 1992), there are subtle differences in the regulation of sMMO activity by protein B. In *M. capsulatus* (Bath), the presence of component B is essential for enzymatic activity, converting the sMMO from an oxidase to an oxygenase (Green and Dalton, 1985). In the absence of protein B, methane is not oxidised, but dioxygen is reduced exclusively to water. In the absence of methane, protein B shuts down the flow of electrons to the hydroxylase, resulting in the reduction of dioxygen to water and in the fully functional complex, methane is oxidised to methanol (Green and Dalton, 1985). However, in *M. trichosporium* OB3b protein B appeared only to stimulate methane oxidising activity since methane oxidation was observed in the absence of protein B (Fox et al., 1989).

Purified protein B from *M. capsulatus* (Bath) is unstable and requires protease inhibitors to minimise its degradation to an inactive form, protein B′ (Pilkington and Dalton, 1990). The cleavage of protein B may be important in regulating the amount of active protein B present in the cell by regulating electron transfer or allowing protein B to maintain a certain conformation (possibly a dimer), necessary for its activity. This could be particularly important in controlling the activity of the sMMO complex in response to the supply of methane to the cell.

The genes encoding sMMO have been cloned and DNA sequenced from *M. capsulatus* (Bath) (Stainthorpe et al., 1989, 1990), *M. trichosporium* OB3b (Cardy et al., 1991) and *Methylocystis* sp. M (McDonald et al., 1997). The gene encoding protein B from *M. capsulatus* (Bath) has been functionally expressed in *Escherichia coli* (West et al., 1992).

The work presented here was undertaken to determine the cleavage site(s) in the protein and to elucidate the roles of protein B and the truncated forms within the sMMO complex.

MATERIALS AND METHODS

Growth conditions. *E. coli* cells were grown at 37°C in Luria-Bertani broth (Maniatis et al., 1982) except strain K38 containing pGP1-2 which was grown according to the method of West et al. (1992). Growth of *M. capsulatus* (Bath) was carried out as previously described (Colby and Dalton, 1978).

Protein purification. Purification of the three components of sMMO from *M. capsulatus* (Bath) were undertaken as described by Colby and Dalton (1978). Purification of protein B from *E. coli* BL21 (DE3) transformed with pEB51 and *E. coli* strains transformed with pEB51-pGP1-2, was undertaken as explained previously (West et al., 1992). To purify glutathione *S*-transferase (GST) fusion proteins from *E. coli* AD202, cells were pelleted and resuspended in 1/200 culture volume of 25 mM Mops, pH 6.8, and lysed by two passages through a cooled French pressure cell (Aminco) at 137 mPa. Soluble extract was obtained after centrifugation at 50400×*g* for 30 min at 4°C. The supernatant was loaded onto a 10-ml glutathione-Sepharose 4B column (Pharmacia Biotech), equilibrated with 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 6.8 (solution A). After adsorption for 2 min, the column was washed three times with 50 ml solution A. Fusion protein was eluted with freshly prepared 5 mM reduced glutathione (pH 6.8). To elute purified recombinant protein, thrombin was added to the glutathione-Sepharose 4B column (2 ng thrombin/μg fusion protein) after adsorption of fusion protein for 2 min. After 5–15 min at 20°C, pure protein was eluted with 25 mM Mops, pH 6.8.

Preparation of protein B′. Protein B′ was prepared from purified protein B (which always contained a 1:1 mixture of protein B′) by standing the protein mixture at 20°C for 3–4 days, which was sufficient time to allow the complete conversion of protein B to protein B′. Purity of the protein B preparation was assessed by SDS/PAGE.

Enzyme assay. Assays for sMMO activity were performed according to the method of Pilkington and Dalton (1990).

Determination of protein concentration. Protein concentration was assayed by the method of Bradford (1976) using commercially available reagent (Bio-Rad) with bovine serum albumin as the protein standard.

Electrospray ionisation mass spectrometry (ESI-MS). A Fisons Quatro II triple quadrupole mass spectrometer (VG Biotech) calibrated with 20 pmol μl^{-1} horse heart myoglobin (Sigma Chemical Co.) was used for analysis of accurate molecular masses. The electrospray carrier solvent was water/acetonitrile (50:50, by vol.) containing 1% formic acid and was applied at a flow rate of 5 $\mu l\ min^{-1}$. Mass spectra were acquired over the range *m/z* 600–1700 during a 10-s scan, and data were collected in multichannel analyzer mode (MCA). Data processing was performed with MassLynx (VG Biotech) software. Maximum entropy processing of conventional electrospray mass spectra of protein samples was performed using the MaxEnt program incorporated in the VG MassLynx software.

Surface plasmon resonance. Carboxymethylated dextran was activated for covalent immobilisation of hydroxylase or reductase proteins by derivatisation with *N*-hydroxysuccinimide, mediated by *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide. Gel-immobilised protein solutions containing 10 μg hydroxylase or reductase in a volume of 200 μl were attached to the BIAcore sensor chip surface (Pharmacia Biosensor) in 10 mM sodium acetate, pH 4. A range of concentrations of proteins B and B′ of 10^{-4}–10^{-5} M were prepared in 25 mM Hepes, pH 7.5, and each protein solution was allowed to flow over the immobilised hydroxylase or reductase proteins. Complex formation was analysed using the BIAlogue software. The dissociation value of the complex was measured by replacing the protein-B-containing solution with buffer.

N-terminal sequencing of proteins B and B′. Samples of purified protein containing equal amounts of proteins B and B′ were blotted onto a poly(vinylidine fluoride) membrane (Immobilon P, Millipore). The blotted protein was visualised by staining with Coomassie blue and destained with 1:4:5, acetic acid/methanol/water (by vol.). The membrane was air dried and bands corresponding to proteins B and B′ were excised from the membrane for sequence analysis. The first 18 N-terminal amino acids were sequenced for both proteins.

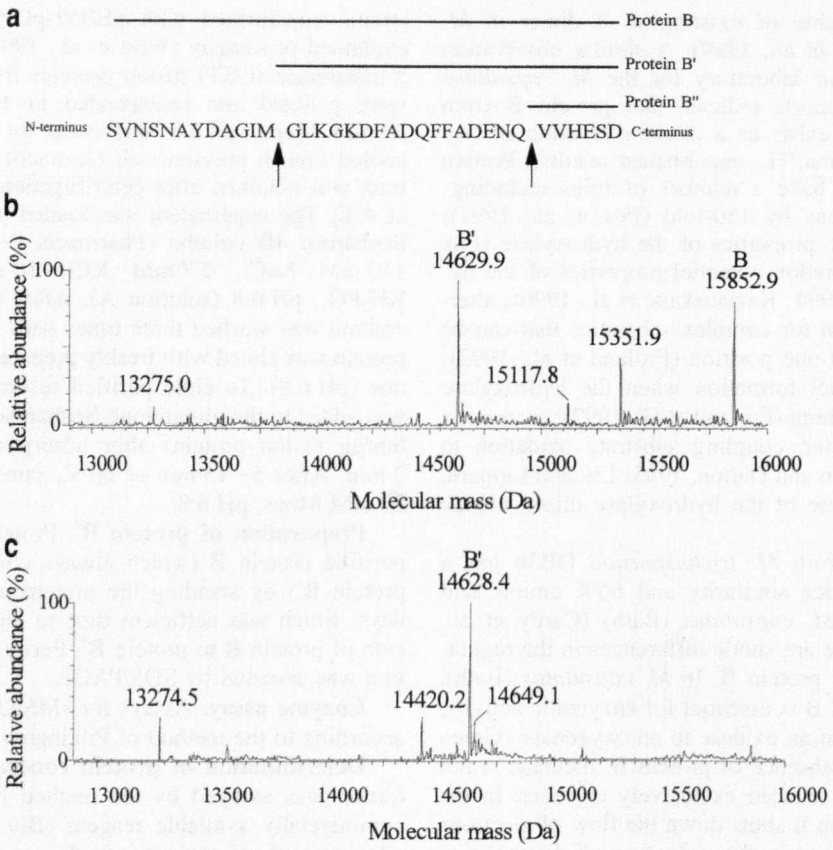

Fig. 1. Cleavage of protein B and ESI-MS of proteins B′ and B″. (a) Cleavage of protein B (indicated by arrows) results in protein B′ and B″ formation. The first six amino acids of protein B″ are shown. ESI-MS of (b) protein B and (c) protein B′ from *M. capsulatus* (Bath) confirmed the molecular masses of protein B and B′ as 15852.6 ± 0.4 Da and 14629.5 ± 0.3 Da, respectively.

Construction of plasmids. pEB51 consisted of a 1.7-kb *Pst*I fragment containing *mmoB*, the 3′ end of *mmoY* and most of *mmoZ* in pT7-5 (West et al., 1992). pGEX-WTB was constructed by PCR amplification of *mmoB* with primer pB-*Bam*HI (5′-GGT GGA TCC ACG **ATG** AGC GTA AAC AGC AAC GCA-3′, start codon in bold and *Bam*HI restriction site underlined) and primer pB-*Eco*RI (5′-GGC GAA TTC TAA GCG TGA TAG TCT TCG AG-3′, *Eco*RI restriction site underlined). Reactions were carried out in a Perkin Elmer 480 DNA thermocycler with 30 cycles of 92 °C, 1 min, 55 °C, 1 min, and 72 °C, 1 min, with a final extension at 72 °C for 5 min. After restriction endonuclease digestion with *Bam*HI and *Eco*RI, the product was ligated into *Bam*HI-digested and *Eco*RI-digested pGEX-2T (Pharmacia Biotech), which resulted in pGEX-WTB. PCR-amplified *mmoB* was sequenced to ensure fidelity of the PCR.

Electroporation. DNA was electroporated into *E. coli* using a Bio-Rad Gene Pulser and Pulse Controller at 200 Ω and 25 μF.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the Unique-Site-Elimination mutagenesis kit (Pharmacia Biotech) according to the manufacturers instructions. A *Nar*I−*Nhe*I selection primer (5′-GCG TAT TGG GCG CTA GCG TGG TTT TTC TTT TCA CC-3′) was used to eliminate the unique non-essential *Nar*I restriction site in pGEX-2T, serving as the basis of selection for mutated plasmids. A target mutagenic primer (5′-GTC CTT GCC TTT CAG CTG CAT GAT GCC GGC GTC-3′) was used to introduce a Gly to Gln mutation in protein B. The gene mutation was confirmed by DNA sequencing.

Production of recombinant protein B in *E. coli*. Typically, cells were induced after they had grown to an A_{540} of 0.5 by addition of 0.1−1 mM isopropyl β-D-thiogalactopyranoside and shaking was continued for 5 h. Growth temperatures and isopropyl β-D-thiogalactopyranoside concentrations were optimised to reduce insoluble protein expression in the form of inclusion bodies.

SDS/PAGE and Western blotting. SDS/PAGE was performed with a Novex X-Cell II apparatus. Pre-cast 4−20% (mass/vol.) gradient gels (Novex) and 12% (mass/vol.) acrylamide gels (Laemmli, 1970) were stained with Coomassie brilliant blue. Molecular masses were calculated using Mark 12 wide-range protein standards (Novex). Western blotting was performed with a Novex Blot Module (Novex) and Hybond C nitrocellulose (Amersham). Antibodies were raised as previously described (West et al., 1992).

RESULTS

Identification of cleavage site. Protein B always co-purified with the truncated form B′, at an approximate ratio of 1:1. Attempts to separate proteins B and B′ using a wide range of chromatographic techniques failed to separate the two forms of the protein. However, proteins B and B′ could be separated as distinct bands on SDS/PAGE gels and so these were electro-eluted onto, then excised from, a poly(vinylidene difluoride) membrane for sequence analysis to determine the precise cleavage site of protein B.

N-terminal amino acid sequencing of the first 18 amino acids of proteins B and B′ revealed that protein B lacked the N-terminal Met, predicted from the DNA sequence, whereas the truncated form (B′) lacked an additional 12 amino acids from the N-terminus (Fig. 1a). This confirmed that protein B′ arose from

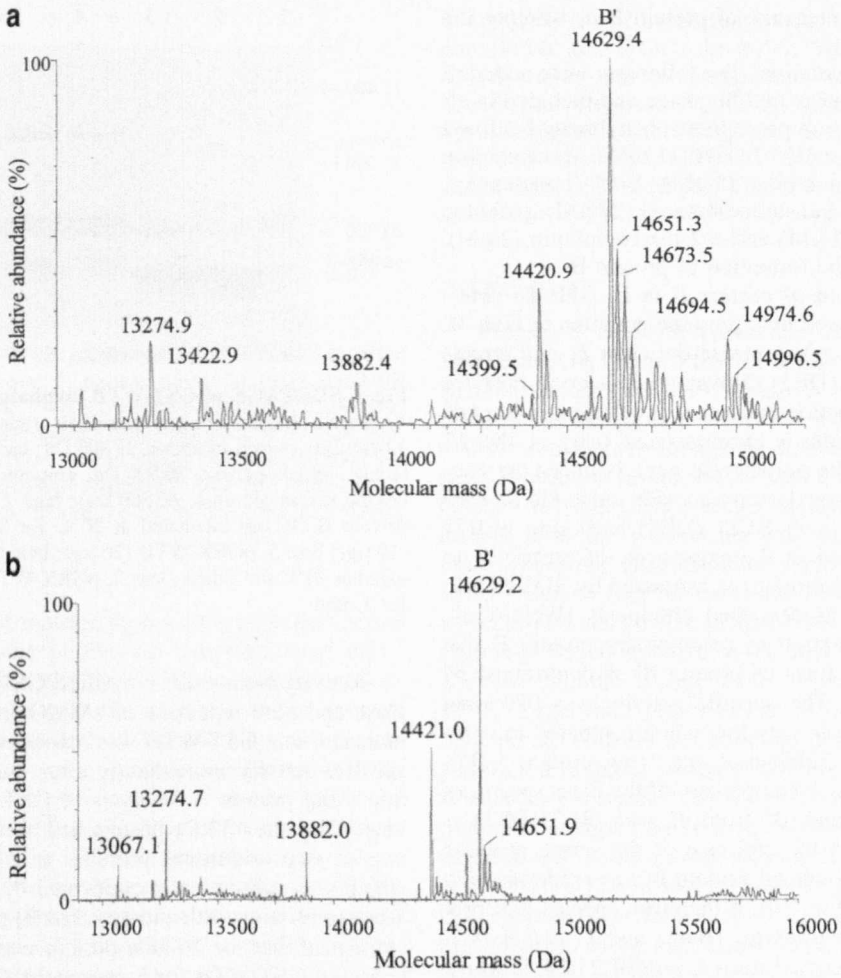

Fig. 2. ESI-MS spectrum of purified protein B from *E. coli* BL21 (DE3). (a) Spectrum in the absence of $PhMeSO_2F$ and (b) in the presence of $PhMeSO_2F$. Protein B′ was detected in both cases and so $PhMeSO_2F$ does not inhibit the truncation of protein B. Complete degradation of recombinant protein B to B′ suggests that it is less stable than protein purified from *M. capsulatus* (Bath).

cleavage between Met12 and Gly13 at the N-terminus of protein B and was not due to a C-terminal cleavage as previously published (Pilkington et al., 1990). Protein B′ has not been reported in *M. trichosporium* OB3b which may be attributed to the Met12-Gly13 site in *M. capsulatus* (Bath) being replaced by a Met12-Gln13 site in *M. trichosporium* OB3b.

ESI-MS analysis of purified proteins B and B′. The ESI-MS spectrum of a preparation of protein B (a mixture of B and B′) from *M. capsulatus* (Bath) showed the presence of two main peaks with molecular masses of 15852.6 ± 0.4 Da and 14629.5 ± 0.3 Da, corresponding to proteins B and B′ respectively (Fig. 1b). However a difference in molecular mass of 34 Da between the predicted value for the DNA sequence data and the measured value was observed. This difference could be rationalised by assuming that the 12th amino acid residue at the N-terminus was Ile and not Phe as indicated from the DNA sequence (Stainthorpe et al., 1989, 1990). Subsequent N-terminal sequencing analysis verified this assumption.

Further cleavage products. In a protein B′ preparation a cleavage product with a molecular mass of 14420.2 Da that accounted for about 25% of the sample relative to protein B′, was observed (Fig. 1c). This would result from cleavage at Met12-Gly13 and Tyr138-His139 and could account for earlier observations (Pilkington et al., 1990) that the cleavage occurred at the C-terminus of the protein. Occasionally, in a purified protein preparation containing proteins B and B′, a third band designated B″ was identified. Protein B″ accounted for about 15% of the sample relative to protein B′ and was thought to be a further breakdown product of protein B′. Protein B″ was found to have a molecular mass of 12709.93 ± 0.02 Da by Fourier transform ion-cyclotron resonance mass spectrometry (FTICR-MS) (data not shown). Cleavage between Gln29-Val30 would result in an expected mass for protein B″ of 12709.47 Da and so protein B″ was probably due to loss of 29 amino acids from the N-terminus of protein B.

Interactions of proteins B and B′ with other components of the sMMO system. Samples with increasing amounts of protein B′ showed a decreasing activity in the standard sMMO assay and samples containing 100% B′ were totally inactive. However, addition of increasing amounts of protein B′ were not found to inhibit activity of the complete sMMO system (i.e. the hydroxylase, reductase, protein B, $NADH_2$, O_2 and propylene). Surface plasmon resonance was used to investigate the interactions of proteins B and B′ with the hydroxylase and reductase components. Rates of formation and dissociation of complexes formed between the hydroxylase (A), reductase (C) and proteins B and B′ were used to calculate stability constants for A-B ($9.0 \times 10^5\ M^{-1}$); A-B′ ($3.0 \times 10^5\ M^{-1}$); C-B ($3.1 \times 10^7\ M^{-1}$) and C-B′ ($3.3 \times 10^5\ M^{-1}$).

Inhibition of protein B′ formation. Attempts were made to determine whether an extrinsic (or possibly contaminating) pro-

tease was responsible for cleavage of protein B or whether the reaction was self catalysed.

Addition of protease inhibitors. The following were added to whole cell suspensions prior to cell breakage and included in all buffers during the purification procedure: phenylmethylsulfonyl fluoride ($PhMeSO_2F$) (1 mM), EDTA (1 mM), benzamidine (1 mM), soybean trypsin inhibitor (5 μM), E-64 (trans-epoxysuccinyl-L-leucylamide-(4-guanadine)butane) (28 μM), pefabloc SC (4 mM), pepstatin A (1 μM) and α-2-macroglobulin (2 μM). None of these prevented the formation of protein B′.

Heterologous expression of protein B in E. coli. To determine if protein B was cleaved by a protease common to both *M. capsulatus* (Bath) and *E. coli*, protease-deficient *E. coli* strains were used. *E. coli* BL21 (DE3) (Novagen) is a good host for recombinant protein production because it is deficient in *lon* and *ompT* proteases and contains a chromosomal copy of the T7 RNA polymerase gene. The polymerase gene is under the control of an isopropyl β-D-thiogalactopyranoside inducible *lacUV5* promoter. Induction of *E. coli* BL21 (DE3) harboring pEB51 (West et al., 1992) resulted in the expression of protein B to about 15% of the total cell protein, as estimated by SDS/PAGE. Purification of protein B as described previously (West et al., 1992) resulted in a preparation of recombinant protein B that was predominately in the form of protein B′ as determined by SDS/PAGE and ESI-MS. The specific activity was 970 nmol min^{-1} mg $protein^{-1}$ which is very low when compared to wild-type enzyme activities of 7289 nmol min^{-1} mg $protein^{-1}$ (Pilkington and Dalton, 1990). A comparison of the mass spectra of recombinant proteins B and B′ from *E. coli* BL21(DE3) is shown in Fig. 2a. Even in the presence of the serine protease inhibitor $PhMeSO_2F$, recombinant protein B was predominantly in the truncated form B′ (Fig. 2b). It therefore appears that protein B purified from *M. capsulatus* (Bath) and *E. coli* K38 is more stable than protein purified from *E. coli* BL21(DE3) where the protein was observed by ESI-MS to be present predominantly in its truncated form, B′.

pEB51 (West et al., 1992) was also transformed into three other *E. coli* strains deficient in *lon* and/or *ompT* proteases, UT5600 (McIntosh et al., 1979), SG20252 (Trisler and Gottesman, 1984) and AD202 (Nakano et al., 1994). SDS/PAGE of soluble extracts followed by Western blotting with anti-(protein B) from *M. capsulatus* (Bath) indicated that protein B′ was identified in soluble extracts of all *E. coli* strains.

Analysis of whole cell extracts from *E. coli* BL21(DE3) lysed in the presence of SDS showed that protein B′ was not present when compared to cells broken by pressure disruption, where protein B′ was observed. The same experiments were subsequently performed with whole cell lysates and soluble extracts of *M. capsulatus* (Bath). Protein B′ from *M. capsulatus* (Bath) was only present in soluble extracts.

Affinity purification of protein B. If active preparations of protein B, free of B′, were to be achieved, it was apparent that recombinant protein B must be rapidly purified from crude extracts. To achieve this, the gene encoding protein B (*mmoB*) was amplified by PCR and the 410-bp PCR product ligated 'in-frame' with the gene encoding GST of pGEX-2T (Pharmacia), forming pGEX-WTB. This resulted in a fusion protein that could be rapidly purified using glutathione affinity chromatography (Smith and Johnson, 1988). The construct was electroporated into *E. coli* AD202 (Nakano et al., 1994) and expression induced with isopropyl β-D-thiogalactopyranoside. SDS/PAGE of whole cell lysates showed the expression of a 43-kDa fusion protein (GST-WTB). Western blotting with anti-(protein B) from *M. capsulatus* (Bath) confirmed that this 43-kDa polypeptide contained protein B from *M. capsulatus* (Bath).

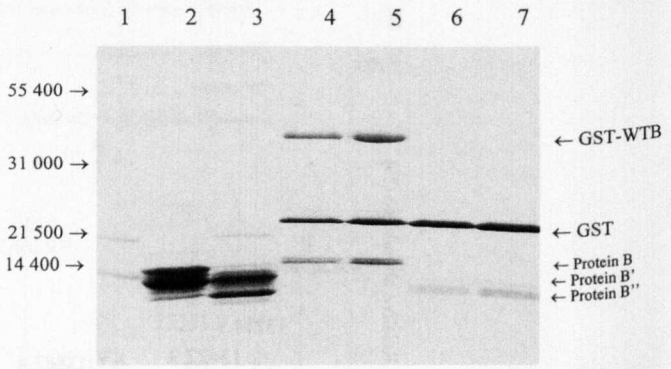

Fig. 3. SDS/PAGE of GST-WTB incubated at 20°C for 3 days. Lane 1, protein molecular-mass standards (aprotinin, 6000 Da; lysozyme, 14400 Da; trypsin inhibitor, 21500 Da; carbonic anhydrase, 31000 Da; lactate dehydrogenase, 36500 Da; glutamic dehydrogenase, 55400 Da; bovine serum albumin, 66300 Da); lane 2, protein B (10 μg); lane 3, protein B (10 μg) incubated at 20°C for 3 days; lane 4, pGEX-WTB (10 μg); lane 5, pGEX-WTB (20 μg); lane 6, pGEX-WTB (10 μg) incubated at 20°C for 3 days; lane 7, pGEX-WTB (20 μg) incubated at 20°C for 3 days.

Activity assays using purified GST-WTB with pure hydroxylase and pure reductase of sMMO from *M. capsulatus* (Bath) indicated that GST-WTB was active even with bound GST. The specific activity immediately after purification was 5700 nmol min^{-1} mg $protein^{-1}$. Analysis of GST-WTB by SDS/PAGE revealed that the 43-kDa protein had been partially cleaved to give rise to two additional proteins at 27 kDa (presumably GST, which was subsequently confirmed by ESI-MS) and at 16 kDa. Western blotting with anti-(protein B) from *M. capsulatus* (Bath) confirmed that the 16-kDa protein was protein B. Further incubation of GST-WTB for 3 days at 20°C showed complete cleavage had taken place since the 43-kDa protein was no longer visible. The protein of 27 kDa had increased in intensity and an additional protein of approximately 15 kDa that cross reacted with anti-(protein B) from *M. capsulatus* (Bath) illustrated complete degradation of protein B to proteins B′ and B″ (Fig. 3). Therefore, although the N-terminus of protein B was protected by fusing it to GST, cleavage still occurred.

The molecular mass of cleaved recombinant WTB following removal of GST by thrombin was 16227.03 ± 7.22 Da as determined by ESI-MS. WTB had a higher molecular mass than wild-type protein B due to the addition of Gly-Ser-Thr-Met at the N-terminus of WTB. To determine the stability of WTB, thrombin cleavage was used to produce a mixture of GST and protein B. This was incubated at 20°C and samples were analysed by SDS/PAGE to determine the extent of B′ formation (Fig. 4). B′ appeared as soon as thrombin was added and by 3 h, greater than 60% of recombinant protein B had degraded to protein B′. [Thrombin did not increase the rate of B′ formation when added to protein B from *M. capsulatus* (Bath).] Immediately after purification, WTB had an enzyme activity of 8800 nmol min^{-1} mg $protein^{-1}$, approximately 15% higher than wild-type protein B from *M. capsulatus* (Bath).

Site-directed mutagenesis of protein B. Protein B′ has not been reported in *M. trichosporium* OB3b which could be attributed to the lack of a Met12-Gly13 cleavage site, where protein B from *M. capsulatus* (Bath) is cleaved to form B′. Therefore, the cleavage site in *M. capsulatus* (Bath) was mutated to the Met12-Gln13 sequence of *M. trichosporium* OB3b. The mutated gene was confirmed by DNA sequencing and the molecular mass of G13Q was verified as 16299.33 ± 1.13 Da. The mutated plasmid was designated pGEX-G13Q.

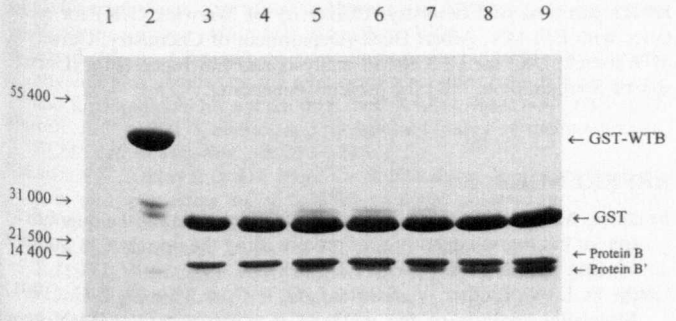

Fig. 4. SDS/PAGE of GST-WTB incubated at 20°C for 3 h, after cleavage with thrombin. Lane 1, protein molecular-mass standards (aprotinin, 6000 Da; lysozyme, 14400 Da; trypsin inhibitor, 21500 Da; carbonic anhydrase, 31000 Da; lactate dehydrogenase, 36500 Da; glutamic dehydrogenase, 55400 Da; bovine serum albumin, 66300 Da); lane 2, GST-WTB (20 μg) (without thrombin); lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 60 min; lane 8, 120 min; lane 9, 180 min.

The enzyme activity of mutated fusion protein B, designated GST-G13Q immediately after purification was 6200 nmol min^{-1} mg $protein^{-1}$. The stability of GST-G13Q was initially determined using SDS/PAGE. As with the wild-type fusion protein, thrombin was used to cleave GST-G13Q and aliquots of separated GST and G13Q were analysed after incubation at 20°C for up to 3 h. Two proteins were identified at about 27 kDa and 16 kDa, corresponding to GST and G13Q, respectively. Protein B′ could not be identified even after 3 h, proving that the Gly to Gln mutation in protein B of *M. capsulatus* (Bath) did prevent the degradation of protein B to B′ (data not shown). Subsequent purification and removal of GST resulted in a specific activity of purified G13Q of 8200 nmol min^{-1} mg $protein^{-1}$.

Effect of mutation G13Q on the stability of protein B. Enzyme assays using WTB and G13Q were performed from 0–8 h during incubation at 20°C and 37°C. At 20°C, GST-WTB had retained 61 ± 4% activity after 8 h in comparison to GST-G13Q which retained 98 ± 3% activity. GST-WTB retained 26 ± 5% activity and GST-G13Q retained 76 ± 4% activity after 8 h at 37°C (Fig. 5a). Stability assays were also performed with pure WTB and G13Q. At 20°C, WTB had retained 46 ± 2% activity after 8 h in comparison to G13Q which retained 57 ± 3% activity. At 37°C, WTB retained 20 ± 4% activity after 8 h in comparison to G13Q which retained 32% ± 3% activity (Fig. 5b). Therefore, GST-G13Q was approximatly 60% more stable than GST-WTB and G13Q was approximatly 20% more stable than WTB.

DISCUSSION

Protein B from *M. capsulatus* (Bath) is cleaved between Met12 and Gly13, such that 12 amino acids are lost from the N-terminus rather than the C-terminus of protein B as previously described (Pilkington et al., 1990). Protein B′ is inactive in the sMMO system even though binding constants determined using surface plasmon resonance, indicated that protein B′ was able to bind to the hydroxylase and reductase components. Binding of protein B′ to the hydroxylase was also determined using direct electrochemistry (Kazlauskaite et al., 1996). Therefore, loss of sMMO activity may be due to the inability of protein B′ to cause a conformational change in the hydroxylase diiron centre, unlike protein B which is active in the sMMO system and does cause a conformational change in the hydroxylase (Davydov et al., 1997). Expression of protein B in various strains of protease-deficient *E. coli* did not alter the degradation of protein B. In addition, a range of protease inhibitors did not affect B′ formation in *M. capsulatus* (Bath) or *E. coli*. Trypsin proteolysis of protein B analysed by N-terminal sequencing (Smith, D. D. S., unpublished observations) showed that trypsin does not cleave at Met12-Gly13 and so a trypsin-like protease is not involved in the cleavage of protein B. Since cleavage occurs between identical residues in *M. capsulatus* (Bath) and *E. coli*, if a protease is responsible for the truncation of protein B then it must be by a highly active and resistant protease common to both of these organisms. Alternatively, an autocatalytic mechanism may be responsible for protein B truncation.

SDS/PAGE and Western blotting of whole cells of *M. capsulatus* (Bath) and *E. coli* expressing recombinant protein B revealed that the truncated form of protein B was not present. Protein B′ can be detected in soluble extracts of *M. capsulatus*

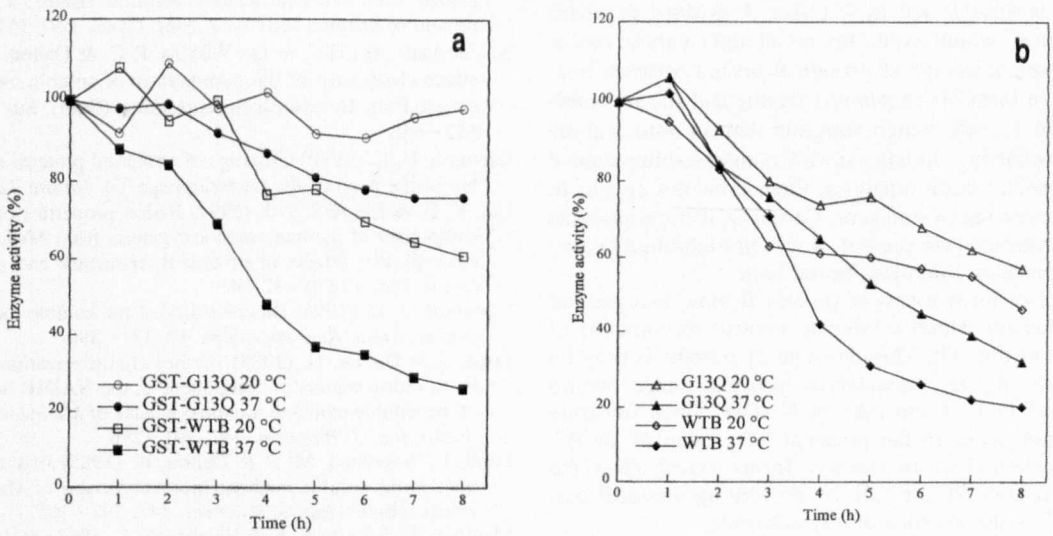

Fig. 5. sMMO propylene oxidation stability assays of (a) GST-WTB and GST-G13Q (b) WTB and G13Q incubated for 8 h at 20 and 37°C. sMMO propylene oxidation assays were performed by adding saturating amounts of hydroxylase and protein C to preparations of 8 μM recombinant protein B. Enzyme activity is the activity remaining after incubation (compared to $t = 0$). 100% activity represents 5700 nmol min^{-1} mg $protein^{-1}$ (GST-WTB), 6200 nmol min^{-1} mg $protein^{-1}$ (GST-G13Q), 8800 nmol min^{-1} mg $protein^{-1}$ (WTB) and 8200 nmol min^{-1} mg $protein^{-1}$ (G13Q).

(Bath) and *E. coli*. Degradation of protein B has been detected in whole cells of *E. coli* expressing protein B using Western blotting, after induction with isopropyl β-D-thiogalactopyranoside for approximately 24 h (unpublished observations). It is not known if this is simply due to degradation of the recombinant protein or whether a specific Met12-Gly13 cleavage is responsible. However, this does indicate the possibility of protein B truncation occurring within whole cells.

Expression of protein B as a GST fusion protein resulted in preparations of protein B with low levels of B′ formation and specific activities comparable, if not higher, than protein B purified from *M. capsulatus* (Bath). At the C-terminus of protein B, a Met130-Gly131 site is found but is not cleaved, presumably because it remains folded within the protein. Similarly, inhibition of protein B degradation may be expected in purified preparations of GST-WTB if the N-terminus of protein B was folded within the fusion protein. However, GST-WTB was cleaved to release protein B′ suggesting that the N-terminus of protein B is more susceptible to degradation than the C-terminus or that the N-terminus does not fold within the protein. Enzyme activity was observed for GST-WTB when purified proteins A and C were used to reconstitute the sMMO protein. As soon as thrombin was added to release WTB from GST, protein B′ was identified by SDS/PAGE. However, alteration of Gly13 to Gln13 enhanced the stability of recombinant protein B preparations. This proved that the Met12-Gly13 cleavage site at the N-terminus of protein B was correctly identified since protein B′ was not observed by SDS/PAGE of protein G13Q. Continued incubation of protein G13Q at 20 or 37°C however, did result in protein B′ formation.

The existence of a truncated form of protein B has also been observed in *Methylocystis* sp. M (Uchiyama, H., unpublished results) due to cleavage between Gln29 and Val30, resulting in the loss of 29 amino acids from the N-terminus. These residues also occur at the same positions in protein B from *M. capsulatus* (Bath) and are cleaved to release protein B″. Clearly, identical cleavage sites in two different methanotrophs is not coincidental and represents another level of control of protein B activity. The ultimate significance of this observation must await detailed *in vivo* studies.

It has not been established whether the formation of protein B′ is the result of a separate protease-mediated cleavage, an autocatalytic cleavage process or a combination of these mechanisms. Purified protein B left at 4°C for 3–4 days degrades entirely to protein B′ which would favour an autocatalytic mechanism. Furthermore, cleavage of protein B occurs between residues 12 and 13 in both *M. capsulatus* (Bath) and the recombinant protein B in *E. coli*, which suggests that an autocatalytic process may be occurring. Judging from Coomassie-blue-stained gels and mass spectroscopic analysis, there does not appear to be another enzymatic species present. Certainly, if trace amounts of an extrinsic protease were present, it was not inhibited by any of the standard protease inhibitors tested here.

The existence of three forms of protein B may be a general regulatory mechanism which is able to control the amount of active protein B in the cell. The cleavage of protein B may be due to activation of the autocatalytic process under certain conditions such as lack of substrate or NADH. Since substrate oxidation does not occur in the presence of protein B′ or B″, proteolysis of protein B to the inactive forms would allow the conservation of energy for the cell, by preventing wasteful oxidation of NADH in the absence of any substrate.

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